FACTORS INFLUENCING THE INCIDENCE OF DIETARY HEMORRHAGIC DISEASE IN CHICKS

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TWO FIGURES

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Evidence for the existence of a new fat-soluble vitamin required by the chick for the prevention of hemorrhagic tendencies and increased blood clotting time has been reviewed in a previous paper (Almquist and Stokstad, '35). Examples of chicks afflicted with hemorrhages as a result of a deficiency of this vitamin are shown in figures 1 and 2. The anti-hemorrhagic vitamin has been concentrated to a state of high potency, about 2 parts of the concentrate per million parts of diet being adequate (Almquist, '36 a). The purity of this concentrate has been increased about four times by distillation under high vacuum (Almquist, '36 b).

It appeared necessary, for the purpose of standardizing assay procedures for the anti-hemorrhagic vitamin, to study certain possible causes of variation in the incidence of this disease. That the exposure of ingredients of the diet to the action of the microorganisms can cause the development of anti-hemorrhagic activity in such materials has already been shown (Almquist and Stokstad, '35). A possible similar synthesis of this factor in the fecal matter of chicks has been investigated. In addition, the transfer of the anti-hemorrhagic vitamin from the diet of the hen to the chick has been studied.

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METHOD AND RESULTS

White Leghorn chicks were kept in electrically heated, wire-floored, all metal brooders. The number of chicks in each experimental group varied from ten to fifteen. The basal diet used was as follows:

Ether extracted fish meal	17.5
Ether extracted dried brewer's yeast	7.5
Salt plus small amounts of cuprie and ferrous sulphates	1.0
Cod liver oil	1.0
Ground polished rice	73.0

This diet contains approximately 18.2% protein, 2.6% ether extract, 4.9% ash, 0.9% calcium and 0.5% phosphorus. When supplemented with the anti-hemorrhagic vitamin, it allows chicks to maintain good health and to grow at normal or better rates. When not so supplemented, this diet causes severe hemorrhagic symptoms within 3 weeks time and heavy mortality, but the growth rate of chicks is not directly affected until the hemorrhagic condition becomes severe.

Droppings from chicks 4 weeks old which had received only the basal diet were collected at 24-hour intervals and dried by immersion in several changes of ethyl alcohol. The dried droppings were then extracted continuously with ethyl ether. The alcohol and ether extracts were mixed and diluted with a large volume of water. The ether layer was drawn off and concentrated.

When incorporated in the basal diet, this fecal extract was found adequate as a source of the anti-hemorrhagic vitamin at a level as low as the equivalent of 5% of dry fecal matter in the diet, and was found marginal at a 1% equivalent level. The extracted fecal residue gave no evidence of protection at a 5% level. The results of this experiment proved that the anti-hemorrhagic vitamin was present in appreciable quantities in the droppings of chicks which were given a diet free of the vitamin. The vitamin was evidently synthesized by bacterial action either after the droppings were voided or within the lower portions of the digestive tract where absorption could not take place readily, or both.

A second collection of droppings from a similar group of chicks was made by allowing them to fall directly into a tray containing 1% phenol in aqueous solution in order to inhibit further bacterial action. The droppings were extracted as before. The final ether solution was washed several times with dilute sodium hydroxide solution and with water.

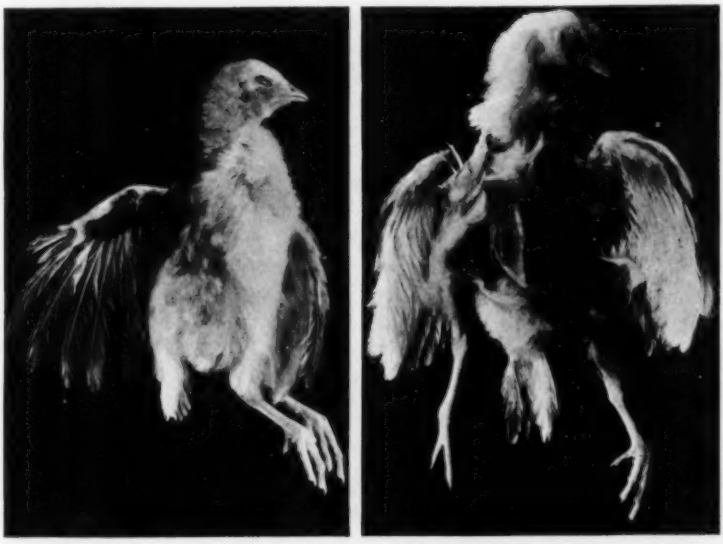

Fig. 1 Subcutaneous hemorrhage on right wing. Chick 3 weeks old. Weight 170 gm.

Fig. 2 Intra-muscular hemorrhages on inside of right leg, left side of breast and left shoulder. Chick 4 weeks old. Weight 245 gm.

As a source of the anti-hemorrhagic vitamin, the ether solution was found adequate at an equivalent level of 8%, marginal at 4% and inadequate at 2%. Presence of the vitamin in this preparation was thus demonstrated, however, the potency was apparently lower than that of the first extract. Because of the high stability of the anti-hemorrhagic vitamin it is unlikely that any of it was destroyed by this treatment.

To obtain evidence on the possible transference of the anti-hemorrhagic vitamin from the diet of the hen to her chicks, eggs from eight pens of laying hens having different levels of dried alfalfa in their diets were hatched. The various diets given the hens approximated normal egg production diets except for the omission of dried alfalfa in certain cases. No supplemental green feed was used in any pen. The chicks from these pens were fed only the basal diet. Records were kept of the number of days lived by each chick and of incidence of hemorrhagic symptoms. The data obtained are given in table 1.

TABLE 1

PEN	PERCENTAGE OF DRIED ALFALFA IN DIET OF HENS	PERCENTAGE INCIDENCE OF HEMORRHAGES IN CHICKS			PERCENTAGE MORTALITY OF CHICKS			AVERAGE NUMBER OF DAYS LIVED PER CHICK
		Weeks			Weeks			
		4	8	12	4	8	12	
141	0	77	94	94	82	100	100	24.5
142	10	13	22	39	35	45	70	50.7
143	0	35	65	94	47	70	100	34.8
144	2½	12	38	43	19	67	76	50.3
145	0	40	80	80	50	100	100	31.3
146	5	13	33	60	13	40	73	55.6
147	0	45	72	83	61	83	100	25.2
148	5	24	60	60	28	75	80	42.6

In this experiment dried alfalfa which is rich in the anti-hemorrhagic vitamin was the principal source of this vitamin given the hens. There was a distinct relation between the presence of this supplement in the diet of the hens and the results obtained with their chicks. In all cases, where alfalfa was fed to the hens the chicks developed hemorrhagic symptoms less readily and survived for a significantly greater length of time. In fact, several birds in these four lots were still alive at the conclusion of the experiment. The variation in the level of alfalfa from 2½ to 10% did not seem to affect the results.

Presence of the anti-hemorrhagic vitamin in egg yolk was demonstrated by feeding hard boiled whole egg yolk at the rate of one-fifth yolk per chick per day. This quantity gave

ample protection. The egg albumen, similarly fed, gave no protection. In a previous paper (Almquist and Stokstad, '35) we reported that the vitamin was not present in the non-saponifiable fraction of yolk fat. It is now known that the vitamin was probably destroyed during the saponification procedure (Almquist, '36).

The vitamin does not seem to be stored in the livers of young chickens (about 8 weeks of age) raised on normal diets containing 5% of dried alfalfa. Liver tissue from such birds was dried in vacuum and fed at 5 and 10% levels as a supplement to the basal diet. In both cases, no appreciable anti-hemorrhagic activity was found.[1]

DISCUSSION

The results on assays of fecal material indicate that chicks used in assays for the anti-hemorrhagic vitamin must not have access to their droppings. The results also indicate that the vitamin may be synthesized, presumably by bacterial action, within the intestinal tract of the chicks but evidently in a region where absorption does not take place. Such synthesis may, however, afford an explanation for cases of spontaneous recovery which we have frequently observed if, under certain conditions, some absorption of the synthesized vitamin may take place. It is also possible that the birds may occasionally consume fecal material before it passes through the wire mesh floor.

Droppings collected under antiseptic conditions appeared to have less potency than those collected at 24-hour intervals, indicating that appreciable further synthesis of the vitamin may have occurred in the latter.

That the diet of the hen influences the incidence of the hemorrhagic syndrome in her chicks is also a demonstrable fact. It has been found desirable to restrict the diet of hens producing chicks for this work to a minimum quantity of dried greens

[1] Since the preparation of this manuscript there has appeared a paper by H. Dam and F. Schonheyder, Biochem. J., vol. 30, p. 897, 1936, in which similar results with liver tissue from young normal chicks are described.

(2.5%) which will permit reasonably satisfactory hatchability and to allow the hens to receive no fresh green feed. Chicks from such a source have given satisfactory response to their diets within 2 weeks.

Precautions must be observed to avoid bacterial action on any or all portions of the diet. For example, feed carried by the chicks to the water troughs must not be allowed to accumulate there and the troughs must be cleaned frequently.

It has been found necessary for uniform results to extract with ether the fish meal and yeast used in the basal diet. When casein is employed it must also be extracted. One of the chief reasons for the use of rice in the basal diet is that it may be obtained in a high state of cleanliness and uncontaminated with weedy or green extraneous plant tissue or diseased kernels. It is free of the anti-hemorrhagic vitamin since extraction of the rice does not increase the severity of the disease.

In certain cases we have obtained indications that alfalfa dust in the atmosphere may influence the results of assays.

Such precautions as have been mentioned are a result of the fact that comparatively very small quantities of the anti-hemorrhagic vitamin are required by the chick.

SUMMARY

1. The anti-hemorrhagic vitamin of the chick is present in fecal matter of chicks receiving none of this vitamin in the diet. It is probably synthesized to some extent within the lower portions of the intestinal tract of chicks and to a further extent in droppings allowed to stand for 24 hours. Chicks employed in studies of this vitamin must not have access to their droppings.

2. The anti-hemorrhagic vitamin is transferred from the diet of the hen to her chick. It is advisable to restrict the diet of hens producing chicks for studies of this vitamin to a minimum level of substances rich in the anti-hemorrhagic vitamin.

3. The anti-hemorrhagic vitamin is present in egg yolk but not in egg albumen. Liver tissue from young normal chicks contains no appreciable quantity of the anti-hemorrhagic vitamin.

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METABOLIC STUDIES OF ESKIMOS IN THE CANADIAN EASTERN ARCTIC

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The data reported here were obtained in 1935 during the Canadian Government Eastern Arctic Patrol on the R. M. S. Nascopie. The examinations included Eskimos in Hudson Bay and Strait, the more northerly parts of Baffin Island and in Devon and Ellesmere Islands as far north as 76° 12′ north latitude—828 nautical miles from the Pole.[1] For reasons stated in the clinical report of this investigation (Rabinowitch, '36) and, as will presently be seen, some of the data are unavoidably fragmentary and are reported for purposes of record and future reference only.

BLOOD CHEMISTRY

The blood analyses include total non-protein nitrogen, urea nitrogen, creatinine, uric acid, amino acid, sugar, chlorides and lipoids. The lipoid studies form a subject matter of another report.

Preparation of materials. None of the tests was completed during the voyage. The work then was confined to collection of the bloods and the necessary treatment in order to preserve them. Folin-Wu (tungstic acid) filtrates were prepared for determination of total non-protein nitrogen, urea nitrogen, creatinine, uric acid and amino acids. To each filtrate were

[1] A portion of the material on the investigation of which this report is based was collected by Dr. C. C. Birchard in Hudson Strait and on the Quebec shores of Hudson Bay.

added a few drops of toluol and all materials were kept cold during the entire voyage. As a further precaution against deterioration, the pH of each filtrate was determined prior to any of the chemical analyses and, without exception, every filtrate was found definitely acid; the pH values ranged between 3.7 and 4.4. The pH determinations were made with the Hellige Standard Model Hydrogen Ion comparator with permanent colored-glass standards.

All chloride determinations were made upon plasma. In each case, the blood was centrifuged as soon as possible after it had been collected and the separated plasma was placed in a tightly corked tube and kept in the refrigerator of the ship during the entire voyage. As all of the laboratory work had to be done on the ship, in a few cases, owing to tide conditions, some hours had elapsed before the bloods could be centrifuged. It is well recognized that unless the plasma and red blood cells are immediately separated from each other after blood has been collected, the concentration of chlorides in the plasma tends to decrease owing to ionic mobility. This possible source of error will again be referred to in the interpretation of the data.

Sufficient quantities of the Folin-Wu filtrates were available for the determinations of blood sugars by a number of methods (Shaffer-Hartmann, Folin-Wu, etc.). The blood sugars to be discussed here, however, were obtained with a slight modification of the Myers-Benedict picric acid method. The reason for this selection will be dealt with in the discussion of the results.

A summary of all of the blood analyses is given in table 1. For purpose of brevity, the number of analyses and maximum, minimum and average values only are recorded.

Discussion of results. Non-protein nitrogen. Bloods of forty-six Eskimos were examined; but in five, after the other constituents had been determined, there were insufficient quantities of the tungstic acid filtrates left for estimation of amino acids. It will be observed that, with the exception of uric acid, though the minimum values of the different constituents are within the generally accepted limits of normality,

the maximum and average values are high. Heinbecker ('28) reported a number of total non-protein nitrogen values in three Eskimos which indicated no retention of nitrogenous products in the blood from the habitual high protein diets; the values ranged between 29 and 43 mg. In one analysis, a very high value—112 mg.—was found; but, in this case, the author suggested that the result was probably due to faulty precipitation of the blood proteins. The observations was also made that by the time the filtrates had been returned to St. Louis for examination all had acquired slight growths of molds. In none of our filtrates was there any suggestion of incomplete

TABLE 1

Blood chemistry of Eskimos (Canadian Eastern Arctic)

CONSTITUTION	NUMBER OF ANALYSES	MILLIGRAMS PER 100 CC.		
		Maximum	Minimum	Average
Non-protein nitrogen	46	64	18	42.0
Urea nitrogen	46	45	14	28.3
Creatinine	46	1.91	1.00	1.55
Uric acid	46	5.26	1.18	3.38
Amino acids	41	10.8	4.0	7.66
Sugar	13	151	110	132
Chlorides (as NaCl)	34	634	560	603

precipitation of proteins and none had any molds. The importance of excluding molds was shown in one of our experiments in which the non-protein nitrogen was determined before and 2 weeks after exposure of a Folin-Wu blood filtrate to room temperature. After the molds had been removed by filtration, 29 only of the 40 mg. of non-protein nitrogen found originally were recovered from the filtrate. Molds, therefore, can account for an appreciable amount of non-protein nitrogen. Heinbecker, however, has the impression that his findings included molds plus filtrate (personal communication).

Impairment of kidney function does not explain our high values. The urinalyses show that these Eskimos were remarkably free of albuminuria and there were no clinical signs to suggest renal disease otherwise (Rabinowitch, '36). These

high values are apparently due to the enormous quantities of meat which these Eskimos eat. When food is plentiful, a healthy adult may eat 5 pounds or more of meat a day. That this is the correct explanation is also suggested from the urinary nitrogen data (see nitrogen metabolism). Meat is often eaten in a putrified state. This, it would appear, is the explanation of the high amino acid values (proteolysis).

Plasma chlorides. In table 1 are also recorded the concentrations of chlorides found in the bloods (plasma) of thirty-four Eskimos. It will be noted that the maximum and average values (expressed as NaCl) are high, in spite of the fact that these Eskimos have no salt in their diets other than that naturally present in the food. As stated, owing to tide conditions, the bloods, in some cases, could not be centrifuged immediately. Therefore, since failure to separate plasma from cells immediately tends to decrease rather than increase the chloride content of plasma, some of the actual chloride concentrations were probably still higher than those found.

URINARY CHLORIDES

Opposed to the high concentrations of the chlorides in the bloods are the extremely low concentrations of chlorides in the urines. This is shown in table 2. It will be noted that the concentrations (also expressed as NaCl) which were found in twenty-two individuals ranged between 0.02 and 0.26% only. Unfortunately, owing to the short time spent at each port during the voyage, it was not possible to collect 24-hour samples of urine, in order to determine total excretions. These Eskimos drink large quantities of water, but dilution of the urines does not appear to be the explanation of these low concentrations for a number of reasons. First, a large part of the ingested water is eliminated by the skin; these people sweat very profusely. This should tend to increase rather than decrease the urinary concentrations of NaCl. That the volumes of urine are no greater among these Eskimos than of people elsewhere is shown by the data reported by Heinbecker ('28, '31, '32). Of forty-six 24-hour specimens, the volumes ranged between

473 and 2995 cc. The average was 1285 cc. Opposed also to undue dilution in our cases are color and specific gravity. None of the urines was pale and the densities ranged between 1.010 and 1.024. The average specific gravity of all of the twenty-two specimens was 1.015. The latter is undoubtedly low compared with the average specific gravity of urine of healthy people elsewhere. Diet is, however, a factor. The specific gravity of urine depends not only upon the amount,

TABLE 2

Sodium chloride and nitrogen concentrations of urines of Canadian Eastern Arctic Eskimos

PLACE	PER CENT		PLACE	PER CENT	
	NaCl	N		NaCl	N
Port Burwell	0.12	0.44	Pangnirtung	0.16	1.54
Port Burwell	0.24	0.59	Pangnirtung	0.20	1.75
Port Burwell	0.24	0.78	Pangnirtung	0.26	1.68
Port Burwell	0.20	0.46	Pangnirtung	0.16	0.91
Port Burwell	0.20	1.09	Pangnirtung	0.16	1.02
Wolstenholme	0.12	0.44	Pangnirtung	0.20	1.52
Wolstenholme	0.02	0.98	Pangnirtung	0.10	1.73
Georges River	0.24	0.82	Pangnirtung	0.10	1.57
Georges River	0.26	0.88	Pangnirtung	0.26	1.90
Craig Harbour	0.14	0.45	Pangnirtung	0.18	1.39
Craig Harbour	0.24	1.11	Pangnirtung	0.08	0.84
Maximum	0.24	1.11	Maximum	0.26	1.90
Minimum	0.02	0.44	Minimum	0.08	0.84
Average	0.18	0.73	Average	0.17	1.48

but also upon the types of solids. These Eskimos are essentially meat eaters and the end product of protein digestion—urea— does not yield as high a specific gravity as corresponding concentrations of NaCl, etc. Combining all of the data, therefore, there is reason to believe that the urinary output of sodium chloride in these Eskimos is very low and this fits in with the dietary habits of these people. They have as stated no salt other than that present naturally in the food which is chiefly meat.

Excretion of water by the skin should, as stated, tend to increase the concentration of NaCl in the urine. Therefore,

unless very large amounts of salt are excreted by the skin, these blood and urine data offer interesting speculation. Unlike urea and other constituents of urine, sodium chloride is a 'threshold' substance—its excretion depends to a large extent upon its concentrations in the blood; and like glucose and other 'threshold' substances, it probably has a threshold because life is incompatible without it. Are the high concentrations in the blood and low excretions in the urine, therefore, the result of a defensive mechanism; that is, an attempt to conserve sodium chloride by raising the renal threshold? It will be observed that the average value, namely, 0.603%, is well above the average renal threshold level of NaCl found among people elsewhere (Aitken, '29).

NITROGEN METABOLISM

In table 2 are also recorded the concentrations of nitrogen found in the above-mentioned twenty-two samples of urine. Since economy of material was not necessary all of the nitrogen determinations were made in duplicate with 5 cc. quantities and with the standard macro-Kjeldhal technic. The data are divided into groups, according to the dietary habits of the natives. In the first column are recorded urines of Eskimos whose diets, though largely native, consist of appreciable quantities of civilized man's foods; whereas, in the second column are recorded the data obtained at Pangnirtung, on Baffin Island, where flour, etc., is available about 2 months in the year only. The diets here are confined largely to the food materials of the environment (seal, etc.). As in the case of sodium chloride, limited significance must be attached to these findings, since high concentrations do not necessarily imply large excretions. The data are, however, very suggestive. It will be observed that the average concentration of nitrogen in the urines of the flesh-eaters was twice that of the natives whose diets are mixed. In one case, the concentration was very high, namely, 1.90%. High concentrations are, however, apparently not uncommon among these Eskimos, in view of the high protein contents of the diets and the relatively low urine

volume output. Thus, even during fasting, Heinlecker ('31) reported daily excretions of 18.90, 24.60 and 21.20 gm. of nitrogen. Since the volumes of urine in the corresponding experiments were 1400, 1380 and 1420 cc., the corresponding concentrations of nitrogen were 1.35, 1.73 and 1.49%, respectively.

The above findings fit in with the interpretation of the high concentrations of non-protein nitrogenous constituents found in the bloods (table 1). Inability to concentrate nitrogen in the urine is an early manifestation of impaired kidney function. Retention of nitrogen in the blood is a late result. These urinary nitrogen data, therefore, support the view that the high concentrations of non-protein nitrogenous constituents of the bloods were not due to impairment of kidney function, but to the habitual use of high protein diets. In our food analyses, for example, it was found that the seal meat contained 3.58% of nitrogen. One pound of such meat would, therefore, alone account for about 15 gm. of nitrogen in the urine and, as stated, when food is abundant, a healthy adult will eat much larger quantities. In these Eskimos, therefore, retention of nitrogen in the blood appears to be a physiological phenomenon.

COPPER METABOLISM

As was shown in the clinical report of this investigation (Rabinowitch, '36) there were a number of cases of polycythaemia among the Eskimos whose dietary habits and activities in general were largely native. Aside from their importance clinically, these cases are of interest with respect to the physiology of formation of haemoglobin. It is now generally believed that copper can, by some catalytic action, influence the rate of formation of haemoglobin and, in general, marine animals have much more copper than land animals. Were the excess quantities of haemoglobin in these cases of polycythaemia, therefore, due to excess intake of copper? This possibility was tested by estimation of the copper contents of the above-mentioned urines. The copper determinations were made by a method previously described by one of the writers

(Rabinowitch, '33) and, as with the urinary nitrogen, the data were divided into two groups according to the dietary habits of the natives. The findings are summarized in table 3. Assuming, therefore, that, as in the case of nitrogen, high concentrations may be taken to be due to large excretions, the data are suggestive. It will be observed that, as in the case of nitrogen (table 2) the highest concentration of copper in the urine and the highest average were found among the flesh-eaters. It should, however, be noted that the averages of both groups are within the limits of the concentrations of copper found in normal individuals elsewhere (Rabinowitch, '33). These averages alone do not, however, exclude copper as a factor in the production of the excess quantities of haemoglobin which were found in these Eskimos. Copper is also

TABLE 3

Showing concentrations of copper (milligrams per liter) in urines of Eskimos

	MIXED DIET	FLESH EATERS
Maximum	0.200	2.000
Minimum	0.050	0.075
Average	0.091	0.390

excreted—and probably to a large extent—by the intestines (bile) and as with other metals (lead, etc.) the urine may not reflect the concentrations in the tissues. 'Balance' experiments would have been helpful; but no preparations were made for this type of work; the polycythaemia was an accidental discovery during the clinical examinations.

CARBOHYDRATE METABOLISM

Glycosuria. In all eighty-eight urines were examined for sugar with Benedict's qualitative reagent, and reducing substances were found in three only. None of these substances, however, fermented with yeast. There was, therefore, no glucose in any of the specimens.

Acetonuria. The above-mentioned eighty-eight urines were also tested for acetone with the sodium nitro-prusside reaction. No acetone was found. All of the urines were preserved

in tightly corked test tubes. The corks were sealed and the tubes were kept cold throughout the entire voyage. Therefore, though there is the theoretical possibility of destruction of beta-oxy and beta-hydroxy-butyric acid, had any been present, there is no reason to doubt the reliability of the acetone data, since exposure of urine to as high a temperature as 38°C. does not affect total acetone values (Heinbecker, '28). However, according to the clinical findings (Rabinowitch, '36) there appears to be no mystery about the absence of ketosis. The Eskimo, in the Canadian Eastern Arctic at least, does not live upon as pure a protein-fat diet as is generally believed. There is a small supply of berries for about 2 months in the year. These Eskimos also relish the stomach contents of the caribou which contain carbohydrates throughout the year, though a large part is probably not utilizable as far as the human being is concerned (celluloses, hemicelluloses, hexosans, pentosans, etc.). One of the writers (IMR) was told that when these Eskimos catch a walrus, they immediately open the stomach and eat the clams which contain glycogen. The livers of all of the animals, with the possible exception of the bear, are eaten and these also contain glycogen. The skin of the whale and of the narwhal are both rich in glycogen and, as stated, when food is abundant, these Eskimos eat enormous quantities of meat which is largely eaten lean; except when food is scanty, blubber is not regarded as a relish. It is also of interest here to note that though some of the Arctic animals have enormous layers of blubber, the accumulation of fat in the musculature are not necessarily large. The meat, therefore, aside from the blubber, may be quite lean. Of the meats which we have examined that of the flipper of the white whale and of the walrus only were found to contain large quantities of fat—17.97 and 12.35%, respectively. The seal meat contained 4.97% of fat.[2] Seal, it should be noted, is regarded very highly, as it also supplies light and heat. It is, therefore, consumed in large quantities. When consideration is given to

[2] Krogh and Krogh are quoted as having found 6 to 10% of fat in seal (Heinbecker, '28).

these facts and also to the potential sugar production from protein (about 58%), it is obvious that the ratio of fatty acid to glucose is below the generally accepted level of ketogenesis. Owing to the short time available during this expedition, the calculations made of the diets of these natives must be accepted subject to modification; but the data suggest that when food is abundant, the average daily diet of the adult Eskimo in the Canadian Eastern Artic consists, approximately, of 40 gm. of carbohydrate (including glycogen) 250 to 300 gm. of protein and about 150 gm. of fat (average FA/G = 1.2). This calculation, it will be observed, fits in with experiences with Greenland Eskimos. Shaffer ('21) cites calculations made by Krogh and Krogh of data collected by Rink, according to which the average diet of these Eskimos consists, approximately, of 54 gm. of carbohydrate, 135 gm. of fat and 282 gm. of protein (FA/G = 1.1).

As stated, these Eskimos have very little salt in their food. This alone might lead toward ketosis. Some of these Eskimos are, however, now perhaps, more exposed to the possibility of alkalosis rather than of acidosis, because of their practice in the use of 'banik.' 'Banik' is essentially a mixture of water, baking soda and flour and the majority of these people have no appreciation whatever of the necessary proportions of these materials in the preparation of this food. When finances permit, the diet may be confined largely to this 'banik' and the daily consumption, at times, may correspond to as much as 25 or more gm. of sodium bicarbonate.

Blood sugar. Though we also had Folin-Wu filtrates, the following observations, as stated previously, are based upon blood sugar data obtained with the Myers-Benedict picric acid method. There are a number of reasons for this selection. Since none of the sugar determinations could be made during voyage, consideration had to be given to the stability of glucose in blood filtrates. The stability of glucose in tungstic acid filtrates is uncertain. From many years of experience in this laboratory, we, however, know the stability of glucose in picric acid solutions. An equally important reason for the selection

of this method was comparability of data. In the interpretation of blood sugar time curves, it is generally accepted that normally the fasting blood sugar ranges between 0.08 and 0.12% and that the peak of the curve—the maximum hyperglycaemic response to ingestion of glucose—is below 0.18%. These standards, it should be noted, were originally derived from experiences with picric acid and not with copper reagents, the values with which are generally lower. Parenthetically, it may here be stated that, in the use of the picric acid technic, all precautions (Rabinowitch, '28) were taken to avoid artificially high values (test of purity of picric acid, etc.).

In table 1 are recorded thirteen sugar determinations of bloods collected at random and it will be observed that, though

TABLE 4

Blood sugar time curves of Eskimos[1]

	NUMBER OF ANALYSES	BLOOD SUGAR (PER CENT)		
		Maximum	Minimum	Average
Before glucose	13	0.181	0.114	0.137
30 minutes after	11	0.250	0.175	0.210
60 minutes after	12	0.258	0.133	0.204
120 minutes after	11	0.192	0.119	0.149
150 minutes after	7	0.161	0.107	0.137

[1] Fifty grams of glucose.

the minimum value is within the generally accepted range of normality, the maximum and average values are high. However, in the interpretation of these data, consideration must be given to the fact that, as with the other blood constituents it was not always possible to collect all of the bloods in the fasting state, owing to the time of the day the ship arrived and the number of hours at the different ports. That the blood sugar of the Eskimo is, however, as a rule, higher than people elsewhere is suggested from the experiences with the thirteen blood sugar time curves shown in table 4. None of these tests was done unless it could be started in the fasting state. Again, for purpose of brevity, the number of tests and maximum, minimum and average values only are given for the different

points of the curves. Another reason, however, for the use of a compositive curve, as the data show, is that it was not possible to obtain complete curves in all cases.

According to the average (composite) curve, it will be observed that the average blood sugar even in the fasting state was high. This also applies to the peak and to the other points of the curve, though it will be noted that, according to the minimum values, some of the points were within the generally accepted limits of normality. Assuming no technical error, these data, therefore, clearly indicate that these Eskimos had difficulty in utilizing glucose in pure form.

The above findings differ to some extent from the four curves reported by Heinbecker ('28). All of the latter were normal, though the Eskimos had followed their usual dietary habits prior to the examinations. Doctor Heinbecker kindly supplied one of the writers (IMR) with a description of the conditions under which these tests were made. All blood sugar determinations were made during the voyage and all reagents were tested before and after the determinations. A finding which is, however, difficult to reconcile with these normal curves is that "the normal height of the 12-hour fasting sugar of the Eskimo subjects is slightly higher than usually observed, averaging about 120 mg. %." This value, it should be observed, agrees very closely with the average found in our thirteen subjects, namely, 0.137%, when consideration is given to the methods used. Heinbecker made use of the Shaffer-Hartmann technic, the values with which are generally 10 to 15 mg. lower than with the picric acid technic. A blood sugar of 0.120% with the Shaffer-Hartmann method is, therefore, definitely high, since it represents the upper limit of normality with picric acid. It would, thus, appear that Heinbecker's Eskimos also had some difficulty to utilizing carbohydrates.

Urines were collected during these tests to correspond to the different points in the curves. There were, therefore, sixty-five specimens and sugar was found in six only. The amounts ranged between very faint traces and traces. In view of the

maximum blood sugars and the high average values at the 30- and 60-minute periods, it is obvious that some of these Eskimos also had a raised renal threshold for glucose.

RESPIRATORY QUOTIENTS

In table 5 are shown ten respiratory quotients obtained in the fasting state and it will be observed that five were lower than 0.7. Had these data been obtained elsewhere, they would have been discarded on the assumption that they were due to undetected technical errors. There were undoubtedly a number of disturbing factors. First, in spite of the excellent cooperation of the natives, conditions were not ideal for respiration work. The weather was cold and the subjects had to be examined in one of the holds of the ship. The latter was well

TABLE 5

Respiratory quotients of Eskimos obtained in the fasting state

SUBJECT	FASTING STATE	SUBJECT	FASTING STATE
1	0.841	6	0.681
2	0.662	7	0.743
3	0.694	8	0.781
4	0.687	9	0.702
5	0.739	10	0.694

ventilated. Second, there was no opportunity to accustom the natives to the tests, because of the short time spent in any one port. However, lack of experience with the test should, because of over ventilation, tend toward artificially high rather than low quotients, owing to excess elimination of CO_2. No difficulties were encountered in the collection of the gas samples. All were preserved in tightly sealed tonometers and all analyses were done in duplicate. Two control samples—one from one of the writers—gave respiratory quotients of 0.830 and 0.813. Drowsiness, a possible cause of low quotients is excluded in these cases. None looked drowsy and drowsiness is incompatible with life in the Canadian Eastern Arctic. These people are very alert and all of the subjects were very interested in their tests. It is of interest here to note that

Heinbecker ('28, '31) observed similarly low quotients in Eskimos as early as the first day of fasting and the data fit in with those obtained by Higgins, Peabody and Fitz ('16) with fat-protein meals.

BASAL METABOLISM

The basal metabolism of the Eskimo appears to be high; the average basal metabolic rate of ten subjects was plus 26%.

TABLE 6

Basal metabolism of Eskimos

SUBJECT	SEX	AGE [1]	HEIGHT	WEIGHT [2]	BODY SURFACE	RESPIRATORY EXCHANGE		TOTAL R.Q.	TOTAL CAL. [3]	CAL. PER SQUARE METER	B.M.R. [4]
						CO_2	O_2				
			inches	*pounds*	*sq.m.*	*liters*	*liters*				
1	M	15	61.0	110	1.47	15.37	18.27	0.841	88.61	60.3	+ 31
2	M	45	63.0	132	1.62	12.52	18.91	0.662	88.61	54.7	+ 42
3	M	30	62.5	140	1.66	11.85	17.08	0.694	80.04	47.0	+ 19
4	F	20	60.0	100	1.39	9.52	13.86	0.687	64.94	46.7	+ 26
5	F	40	57.0	112	1.41	9.67	13.09	0.739	61.90	43.9	+ 22
6	M	55	64.0	136	1.65	10.29	15.11	0.681	70.80	42.9	+ 14
7	M	60	61.0	118	1.51	10.24	13.78	0.743	65.14	43.1	+ 18
8	F	30	63.5	111	1.51	11.90	15.24	0.781	72.78	48.2	+ 32
9	M	35	62.0	104	1.44	11.07	15.77	0.702	73.90	51.3	+ 30
10	M	50	65.0	148	1.74	12.16	17.52	0.694	82.10	47.2	+ 26
A.M.		38	61.9	121.2	1.54	11.46	15.86	0.722	74.88	48.5	+ 26

[1] Approximate (within 5 years).

[2] Adjusted for ounces.

[3] Calculated from calorific values of O_2 according to non-protein R.Q. Calorific value of 1 liter of O_2 at R.Q. less than 0.7 taken as at R.Q. = 0.7.

[4] Du Bois standards.

This is shown in table 6. There are, however, a number of possible errors inherent in the data. First, the basal metabolic rates were calculated from the respiratory quotients and it was assumed that the calorific value of a liter of oxygen with respiratory quotients lower than 0.7 was the same as at 0.7. There is also a possible error in the use of the Aub and Du Bois standards. These may not, and probably do not, apply to Eskimos. It should, however, be noted that with the

use of these low respiratory quotients, we have assumed minimum rather than maximum heat values. The true calorific values of the oxygen consumed may, therefore, have been greater than those used in the calculations. The true basal metabolic rates may, therefore, have been greater than those found.

A priori, one would expect these Eskimos to have high basal metabolic rates. The constant stimulation of the cold weather should, theoretically, tend toward increased muscle tone and basal metabolism is, to a very large extent, a function of active protoplasmic mass. These people are also, as stated, largely carnivorous in their dietary habits. There is, therefore, the added factor of the high specific dynamic action of proteins. A number of these individuals also had polycythaemia. This may also have been a factor, though according to Schaternikoff ('28) the ability of the blood to carry oxygen does not appear to be an important factor. In order, however, that the data may be subjected to further analysis when more is known of the variables mentioned, all of the details of the tests are recorded in the table. The data include age, sex, height, weight, body surface, consumption of oxygen and production of carbon dioxide.

METABOLISM AFTER INGESTION OF FAT

In table 7 are recorded two tests in which an attempt was made to determine the effects of ingestion of fat upon the respiratory quotient and metabolic rate. In each case, the fat meal consisted of 200 cc. of soya bean oil. It will be noted that respiratory quotients lower than 0.7 were observed here also, though in both subjects the quotients in the fasting state were definitely higher. Combining all of the data, it would, therefore, appear than in the Eskimo may be found the solution to the problem of conversion of fat to sugar. It is of interest to note that the increased heat production after each meal corresponded to the specific dynamic action of peoples elsewhere. As with the basal metabolic rates, however, all of the data are recorded in detail for any re-calculations which may be found necessary with a better knowledge of variables.

SPECTROGRAPHIC ANALYSES

Because of a) the alleged absence of arteriosclerosis among Eskimos, b) the high incidence of arteriosclerosis among civilized people, c) the well-known harmful effects of lead upon the cardio-vascular system and d) the fact that small amounts of lead are almost invariably found in urines of perfectly normal individuals, particular attention was paid to lead in this investigation. Because it was suspected that minute

TABLE 7

Metabolism of Eskimos. Effects of ingestion of fat[1]

PERIOD	RESPIRATORY EXCHANGE		R.Q.	HEAT PRODUCTION (CALORIES)[3]	
	CO_2	O_2		Total	Increase above basal
Experiment 1. Subject no. 5. Female. Age 40.[2] Height 57 inches. Weight 112 pounds. Body surface 1.41 sq.m.					
	liters	*liters*			%
Fasting	9.67	13.09	0.739	61.90	
1 hour after meal	9.92	14.53	0.683[4]	68.09	10.0
2 hours after meal	9.87	14.95	0.660[4]	70.05	13.2
3 hours after meal	9.52	13.49	0.706	63.21	2.1
Experiment 2. Subject no. 7. Male. Age 60.[2] Height 61 inches. Weight 118 pounds. Body surface 1.51 sq.m.					
Fasting	10.24	13.78	0.743	65.14	
1 hour after meal	11.42	15.62	0.731	73.63	13.0
2 hours after meal	10.64	15.05	0.707	70.52	8.2
3 hours after meal	8.94	14.58	0.613[4]	68.32	4.9

[1] Two hundred cubic centimeters Soya bean oil.
[2] Approximate (within 5 years).
[3] Calculated from calorific values of O_2 according to non-protein R.Q.
[4] Calorific value of 1 liter of O_2 at R.Q. less than 0.7 taken as at R.Q. = 0.7.

traces only would be found, the urines were subjected to spectrographic rather than to chemical analysis. The spectrograms were made in the physics laboratories of McGill University by Dr. John S. Foster. No lead was found in any of the twenty-four urines examined, though as little as 5×10^{-8} gm. of this metal may be readily detected in 1 cc. of urine by the method employed. This absence of lead is particularly of interest, in view of the fact that, as our data clearly show

(Rabinowitch, '36) the Eskimo does develop arteriosclerosis. In the Eskimo, at least, therefore, some other cause must be sought for this disease.

Urines were also examined spectrographically for copper and, in general, the data agreed with the above-mentioned chemical findings.

According to the spectrograms, there were appreciable quantities of magnesium in some of the urines. The significance of these findings is not clearly understood. It is, however, of interest to note that, unlike all other flesh foods in the Eastern Arctic, seal meat has a purgative action and the Eskimo eats large quantities of seal.

Finally, of biological interest, and, as stated in the clinical report (Rabinowitch, '36) is the fact that no definite evidence of rickets was found in any of the 389 natives examined (there were three suspicious cases). The absence, or at least rarity, of this condition, especially at the latitudes of Clyde River (70° 26′), Pond Inlet (72° 40′), Dundas Harbour (74° 35′) and Craig Harbour (76° 12′) is instructive when consideration is given to the fact that the effectiveness of the sun's rays in the production of vitamin D-like substances decreases toward the Pole. Furthermore, the Eskimo's skin is quite pigmented. This should decrease penetration of the little of the sun's rays which are effective. The reason, however, for the absence of rickets is the practice of nursing infants until they are 2 and 3 or more years old and the fact that the vitamin D-content of seal oil is equal to that of the best cod liver oil.

SUMMARY

The average concentrations of non-protein nitrogenous constituents of the bloods of forty-six Eskimos were found to be higher than of peoples elsewhere and could not be explained by impairment of kidney function nor by conditions generally known to lead to such high values otherwise. Retention of nitrogen in the blood appears to be a physiological phenomenon among these Eskimos and is apparently due to the high protein diets.

The concentrations of chlorides in plasma were determined in thirty-four Eskimos and both maximum and average values were higher than the average renal threshold level of NaCl of peoples elsewhere. In spite of these high values, the concentrations of chlorides found in the urines were extremely low. The possible significance of these findings is briefly discussed.

None of the urines collected under ordinary conditions contained glucose or acetone according to the usual tests. The absence of glucose fits in with the absence of diabetes clinically (Rabinowitch, '36) and to explain the absence of ketonuria it does not appear necessary to seek for some unknown mechanism for utilization of fat. When food is plentiful, fat is not regarded as a relish and the carbohydrate contents of certain foods, the high protein intake and the potential sugar production from protein are such that the ratio of fatty acid to glucose is below the generally accepted level of ketogenesis.

Though there was no glycosuria and no clinical evidence of diabetes mellitus, the average blood sugar of thirteen Eskimos in the fasting state suggests some difficulty in the utilization of carbohydrates. The blood sugar time curves obtained in these individuals following ingestion of glucose clearly indicated that these Eskimos were unable to utilize large quantities of glucose in pure form.

Respiratory quotients lower than 0.7 were found in five of ten individuals in the fasting state and in three of eight tests in two individuals after administration of fat meals and, though conditions were not ideal for this type of work, no sources of error could be detected. The combined data, therefore, suggest that, though there is nothing peculiar about the absence of ketosis, it is still possible that the metabolism of fat in these Eskimos may to some extent be different from peoples elsewhere and that in these Eskimos may be found the solution to the controversy with respect to the conversion of fat to sugar.

The basal metabolic rates of these Eskimos appear to be high. The data fit in with the cold environment which is known to increase metabolism and also with the food habits of these people, in view of the high specific dynamic action of protein.

Urines were examined spectrographically with respect to a number of inorganic elements. No lead was found in any of the twenty-four specimens examined though the sensitivity of the method employed is approximately 5×10^{-8} gm. per cubic centimeter. The significance of these findings with respect to arteriosclerosis is briefly discussed.

The copper contents of the urines, as determined spectrographically, were essentially the same as found by chemical analysis.

According to the spectrograms, the urines contained relatively large quantities of magnesium. The explanation is not clear, but the large amounts of seal meat in the diets and the known purgative action of this meat are suggestive.

The absence or at least rarity of rickets among these Eskimos, in spite of their proximity to the Pole and pigmentations of the skin, appears to be due to the long periods during which infants are breast fed and the fact that the vitamin D-content of seal oil is equal to that of the best cod liver oil.

The above studies were part of a general investigation of the health of Eskimos in Canada's Eastern Arctic. Space does not permit mention of the many whose cooperation made this investigation possible. Due acknowledgment was, however, made in the report to the Dominion Government and in a previous communication (Rabinowitch, '36).

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THE INFLUENCE OF VITAMINS A, B OR D, ANEMIA OR FASTING UPON THE RATE OF FAT ABSORPTION IN THE RAT [1, 2]

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INTRODUCTION

In a preceding communication we reported differences in the rate of absorption of various fats (Steenbock, Irwin and Weber, '36). Of the fats tested halibut liver oil, cod liver oil and butter oil were absorbed the most rapidly. As these fats are distinguished from the others in containing considerable quantities of vitamin A and some vitamin D, it seemed desirable to determine if these vitamins were responsible for the differences.

That vitamins play a role in the absorption of foodstuffs has been suggested previously. For example, there have been published numerous reports to the effect that the secretory and motor activities of the digestive tract are decreased in avitaminosis (Boldyreff, '31; Hagashi, '30; Menville, Ane and Blackberg, '31; Menville, Blackberg and Ane, '29; Mottram, Cramer and Drew, '22; Murakami, '28; Never, '28, '30; Pierce, Osgood and Polansky, '29; Webster and Armour, '33; and Yonemura, '27). Gal ('30) and Kokas and Gal ('29) using technic similar to that used in our experiments have shown that the rate of absorption of protein and carbohydrate

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[2] Published with the permission of the director of the Wisconsin Agricultural Experiment Station, Madison.

is decreased in B avitaminosis and that a yeast extract is capable of stimulating the absorption of sugar and of peptone. On the other hand, the total digestibility of a foodstuff has been stated not to be affected by a lack of any one of the vitamins (Bergeim, '28; St. Julian and Heller, '31; and Sampson, Dennison and Korenchevsky, '32). Because of the natural occurrence of vitamin A in fats a number of investigators have associated vitamin A with fat metabolism. Domagk and Dobeneck ('33) have suggested that vitamin A bears the same relationship to fat metabolism that vitamin D does to calcium metabolism. Our investigations, accordingly, concerned themselves with the effect of vitamins A, B and D on fat absorption. Incidentally, we also determined the effect of anemia and fasting.

EXPERIMENTAL

The rate of absorption of fat was determined by the method previously described by Irwin, Steenbock and Templin ('36), which is a modification of the technic used by Cori ('25) for determining the rate of absorption of sugar. Adult rats were fasted 48 hours, fed 1.5 cc. of melted fat by stomach tube, killed after a definite time interval, and the residual fat in the alimentary canal determined after 4 hours. A 4-hour absorption period was used because this period proved to be the critical one. Both male and female rats were used, as we have shown that the percentage of fat absorbed in a definite time period is the same for both sexes (Irwin, Steenbock and Templin, '36). The fats[3] used were halibut liver oil, a vegetable shortening (fat A), and a vegetable salad oil (fat W).

Vitamin A and fat absorption. To determine if the more rapid rate of absorption of halibut liver oil could be due to its vitamin A content, an absorption test was carried out with oil in which the vitamin A had been destroyed by aeration at 100°C. Aeration was discontinued when the antimony tri-

[3] The fats and oils used were those available commercially. The halibut liver oil was obtained from Parke, Davis & Company. Hydrogenated vegetable shortening A was prepared by hydrogenating cottonseed oil to an iodine number of approximately 70. The saponification number was 196.0. Fat W was a refined cottonseed salad oil.

chloride test was negative, which required 36 hours of treatment. Of this oil only 58% was absorbed in contrast with 70% for the controls (table 1). The mean difference of 12% was significant as it gave a standard deviation of 4.2 (table 2).

Further data were obtained by determining the effect of vitamin additions to the aerated oil. A vitamin A-concentrate was prepared in the following manner. Halibut liver oil was

TABLE 1

The percentage of fat absorbed in 4 hours by rats fed 1.5 cc. of fat

GROUP	FAT AND SUPPLEMENTS	CONDITION OF RATS	NUMBER OF RATS	RANGE IN WEIGHT	FAT ABSORBED
				gm.	%
1	Halibut liver oil	Normal	10	245–345	70 ± 2.0
2	Aerated halibut liver oil	Normal	6	225–315	58 ± 2.0
3	Aerated halibut liver oil + 28,000 blue units of vitamin A per cubic centimeter	Normal	10	240–338	54 ± 2.1
4	Fat A	Normal	11	236–392	54 ± 1.6
5	Fat A + 26,400 blue units of vitamin A per cubic centimeter	Normal	11	275–350	49 ± 1.1
6	Fat A	Avitaminotic A	11	98–134	30 ± 0.9
7	Fat A + 24,200 blue units of vitamin A per cubic centimeter	Avitaminotic A	4	107–140	31 ± 1.6
8	Fat A	Young, normal	5	125–145	56 ± 2.4
9	Fat W	Normal	10	275–382	56 ± 1.7
10	Fat W	Avitaminotic B	11	217–302	45 ± 1.6
11	Fat W	Avitaminotic D	12	100–160	37 ± 2.0
12	Fat A + yeast extract	Normal	5	250–300	49 ± 2.4
13	Fat A + 320 International units of vitamin D per cubic centimeter	Normal	10	280–426	58 ± 1.3
14	Fat A	Anemic	4	90–124	45 ± 1.6
15	Fat A	Reared on limited amount of stock ration	8	103–150	35 ± 1.0
16	Fat A	Fasted 6 days	10	290–365	44 ± 1.6

saponified, extracted with ether, and the ether distilled off in a partial vacuum with nitrogen. The residue was taken up in methyl alcohol and allowed to stand for 12 hours at 1°C. The sterols which crystallized out were filtered off. The methyl alcohol was dissipated in a partial vacuum under nitrogen and the residue dissolved in aerated halibut liver oil to give a concentration of 28,000 blue units per cubic centimeter. Of this mixture the rats absorbed 54 %—only 4% less than the control with no vitamin A addition, which is not statistically significant. Apparently then, in the preceding experiment the

TABLE 2

Significance of the differences in absorption

DIFFERENCE BETWEEN	MEAN DIFFERENCE	STANDARD DEVIATION OF MEAN DIFFERENCE	$\frac{MD}{\sigma MD}$
	%		
Groups (1) and (2)	12	4.2	2.9
Groups (2) and (3)	4	4.3	0.9
Groups (4) and (5)	5	2.9	1.7
Groups (4) and (6)	24	2.7	8.9
Groups (9) and (10)	11	3.5	3.1
Groups (9) and (11)	19	3.9	4.9
Groups (4) and (12)	5	4.3	1.2
Groups (4) and (13)	4	3.0	1.3
Groups (4) and (14)	9	3.3	2.7
Groups (4) and (15)	20	2.7	7.4
Groups (4) and (16)	10	3.3	3.0

lowered rate of absorption of the aerated oil was not due to the destruction of vitamin A per se, but probably was caused by changes in the character of the glycerides induced by oxidation. As other methods of vitamin A removal than destruction by oxidation were not available, we were forced to limit further trials to determinations of the effect of vitamin A when added to an unoxidized fat. Such experiments were carried out with our halibut liver oil concentrate added to a hydrogenated fat (fat A) to a concentration of 26,400 blue units per cubic centimeters. This mixture was absorbed to the extent of 49%. Of the control fat without vitamin A, 54% was absorbed.

The effect of vitamin A-depletion in the animal upon the rate of fat absorption was also determined. Young rats weighing from 60 to 80 gm. were placed on a vitamin A-free diet for 7 weeks. They then weighed from 98 to 134 gm. (average 118 gm.) and showed signs of xerophthalmia. They were fasted 48 hours, then fed 1.5 cc. of fat A. Only 30% of the fat administered was absorbed. Later a group of four vitamin A-deficient rats weighing 107 to 140 gm. (average 124 gm.) was fed the same fat carrying 26,400 blue units of vitamin A per cubic centimeter. The mean percentage of absorption was essentially the same, namely 31%. However, since the vitamin A-deficient rats were smaller than the adult rats previously used as controls, additional control tests were carried out with smaller animals. The rats used for these tests weighed from 125 to 140 gm. (average 133 gm.). They were found to absorb 56% of the fat in comparison with 54% for the adults. It was, therefore, evident that the size of the animals per se was not responsible for the lower values obtained with the vitamin A-deficient rats.

Effect of avitaminosis B. A group of adult rats was fed a vitamin B-deficient diet (Kemmerer and Steenbock, '33) for 5 weeks. At the end of this time they showed symptoms of polyneuritis and had lost on an average 58 gm. in weight. Their weights ranged from 198 to 284 gm. After fasting for 48 hours they were given 1.5 cc. of fat W. Forty-five per cent of the fat was absorbed in 4 hours, whereas the controls, normal adult rats, absorbed 56%. The mean difference (11%) justifies the conclusion that vitamin B-deficient rats do not absorb fat as rapidly as normal animals.

To determine if a yeast extract would stimulate the absorption of fat, five normal rats were fed fat A as before, but 1 cc. of yeast extract was given directly after the fat. The yeast extract was prepared by treating 500 gm. of yeast containing 20 International units of vitamin B per gram with 1500 cc. of 70% alcohol containing 0.5% hydrochloric acid. The mixture was heated to boiling, allowed to stand 2 hours, filtered, and the filtrate concentrated in a partial vacuum at 50°C. The

concentrate was extracted three times with ether to remove the lipoids. Any ether remaining in the concentrate was removed by evaporating in a partial vacuum at 50°C. Rats given 1 cc. of this solution, equivalent to 3.3 gm. of yeast, absorbed 49% of the fat fed, whereas the controls absorbed 54%. The addition of the yeast extract, therefore, did not affect the percentage of absorption over that of the control group (table 2).

Effect of vitamin D. This was determined with young rats weighing approximately 135 gm. (range 100 to 160) which had been fed the Steenbock and Black ('25) rachitogenic diet no. 2965 for 7 weeks when they began to lose weight and had developed definite signs of rickets. Serum from a pooled blood sample from six of these rats contained 4.0 mg. of inorganic phosphorus per 100 cc. The skeletons revealed wide metaphyses and enlarged costochondral junctions. Six rats from this group absorbed fat W to the extent of 37%, whereas normal animals absorbed 56%.

We also determined if vitamin D added to fats would increase the rate of fat absorption in normal rats. To 1.5 cc. of a sample of fat A there was added a sufficient quantity of a standardized preparation of irradiated ergosterol to give a concentration of 320 International units of vitamin D per cubic centimeter. Fifty-eight per cent of this fat was absorbed in 4 hours. Of the control fat 54% was absorbed.

Effect of anemia. Since all of the animals on vitamin-low diets failed to absorb fat so readily as animals on adequate diets and since such animals were generally in a poor physical condition, the absorption of fat was studied in rats in a similar poor physical condition as induced by other means. Among these was the effect of anemia.

Four anemic rats 11 weeks of age, weighing 90 to 124 gm. (average 107 gm.), that had been reared on a milk diet and had 2.4, 3.6, 3.6 and 4.1 gm. of hemoglobin per 100 cc. of blood, respectively, were fed 1.5 cc. of fat A. These animals absorbed 45% of the fat fed. This is significantly less than the amount absorbed by normal animals.

Effect of starvation. Another factor studied was starvation. A group of young rats weighing 60 to 80 gm. was fed a limited quantity of a stock diet (Steenbock, '23) for a period of 6 weeks. At the end of this time they weighed from 103 to 150 gm. This is an abnormally low weight because similar animals fed ad libitum would have weighed from 200 to 300 gm. at this age. These semi-starved animals absorbed 35% of the fat fed, whereas normal rats absorbed 54%.

In a second experiment adult male rats weighing 290 to 365 gm. were fasted for 6 days and then fed 1.5 cc. of fat. Forty-four per cent of the fat was absorbed in 4 hours, which is 10% less than that absorbed by the controls. It is evident that a decreased rate of absorption in avitaminosis may be ascribed secondarily to a poor physical condition rather than to the specific lack of a vitamin or vitamins.

SUMMARY

1. Avitaminotic A, B or D animals absorb fat less rapidly than normal animals, which effect is not specific for any one of the vitamins tested.

2. The addition of vitamins A, B or D to a fat had no effect upon its rate of absorption by normal rats.

3. Anemic animals, those fasted 6 days, or those fed a limited quantity of a complete diet also absorbed fat less rapidly than controls.

4. As the experiments on anemia and semi-starvation showed that the rate of fat absorption was influenced by the nutritional state of the animal, it cannot be concluded that the vitamin deficiency experiments revealed any specific effects of vitamins on fat absorption.

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THE INFLUENCE OF CERTAIN HYDROTROPIC AND OTHER SUBSTANCES UPON FAT ABSORPTION [1, 2]

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INTRODUCTION

Neuberg ('16 a, b) has shown that certain hydrotropic substances are capable of rendering soluble and diffusible, compounds, which of themselves are insoluble in water. The work of Verzar and Kuthy ('29 a, b, c, '30, '31), Verzar ('31), Wieland and Reverey ('24), Wieland and Sorge ('16) and Reinboldt ('27) has shown that bile acids combine with fatty acids, forming complex aggregations which are diffusible and stable within the reaction range prevailing in the intestine. It is quite possible that the rate of absorption of a foodstuff may depend not only on the functional activity of the digestive organs, but on the presence of hydrotropic substances in the food mixture. In this laboratory a variety of substances have been fed with fats in order to determine the effect of these substances upon the rate of fat absorption.

EXPERIMENTAL

Adult rats that had been fasted 48 hours were lightly anesthetized with ether and fed 1.5 cc. of melted fat by stomach tube. Immediately following the fat feeding 1 cc. of a water solution of the substance under investigation was fed, also by stomach tube. After a 4-hour absorption period the rat was killed and the amount of fat which had been absorbed was determined by analyzing the residues in the intestinal tract.

[1] The authors wish to express their sincere appreciation of the financial support of the Lever Brothers Company, which made this work possible.

[2] Published with the permission of the director of the Wisconsin Agricultural Experiment Station, Madison.

TABLE 1

The percentage of fat absorbed in 4 hours by rats fed 1.5 gm. of fat followed by 1 cc. of a solution containing the substance being tested

GROUP	TREATMENT	NUMBER OF ANIMALS	PERCENTAGE OF FAT ABSORBED
	Controls:		
1	Fed 1.5 cc. fat only	11	54 ± 1.6
2	Fed 1.5 cc. fat plus 1 cc. distilled water	10	55 ± 1.9
	Effect of bile. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
3	1 mg. commercial bile salts	5	53 ± 2.4
4	50 mg. commercial bile salts	5	55 ± 1.9
5	500 mg. commercial bile salts	6	19 ± 2.3
	Effect of sodium benzoate. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
6	1 mg. sodium benzoate	6	50 ± 2.0
7	50 mg. sodium benzoate	6	38 ± 2.5
8	500 mg. sodium benzoate	3	14 ± 3.3
	Effect of ethyl alcohol. Fed 1.5 cc. fat plus 1 cc. of a solution containing:		
9	5% alcohol	7	51 ± 1.7
10	15% alcohol	6	47 ± 1.8
11	40% alcohol	8	27 ± 5.8
	Effect of peptone, sucrose and a mixture of peptone and sucrose. Fed 1.5 cc. of fat plus 2 cc. of a solution containing:		
12	1 gm. peptone	5	27 ± 1.5
13	1 gm. sucrose	5	33 ± 2.4
14	1 gm. peptone and 1 gm. sucrose	6	25 ± 2.6
	Effect of KCl. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
15	0.2 mg. potassium chloride	10	48 ± 1.5
16	20.0 mg. potassium chloride	10	55 ± 1.2
17	100.0 mg. potassium chloride	7	34 ± 2.6
	Effect of $CaCl_2$. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
18	0.2 mg. calcium chloride	6	54 ± 2.5
19	20.0 mg. calcium chloride	6	54 ± 3.2
20	100.0 mg. calcium chloride	6	26 ± 2.9
	Effect of NaH_2PO_4 and glycerol. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
21	2 mg. NaH_2PO_4	6	47 ± 1.2
22	10% glycerol	7	43 ± 2.5
	Effect of sodium glycerophosphate. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
23	2 mg. sodium glycerophosphate	7	47 ± 1.3
24	50 mg. sodium glycerophosphate	7	38 ± 2.0
25	500 mg. sodium glycerophosphate	7	29 ± 2.7

The animals were male and female rats from our stock colony and were 4 to 6 months of age. We have shown previously that there is no significance difference between the percentages of fat absorbed by male and female animals. For details of the method used and for tests dealing with the accuracy of the technic, etc., the reader is referred to the first paper of this series (Irwin, Steenbock and Templin, '36).

TABLE 2

Significance of differences in absorption

DIFFERENCE BETWEEN	MEAN DIFFERENCE	STANDARD DEVIATION OF MEAN DIFFERENCE	$\frac{MD}{\sigma MD}$
	%		
Groups (1) and (2)	1	3.8	0.3
Groups (1) and (5)	35	4.1	8.5
Groups (1) and (6)	4	4.0	1.0
Groups (1) and (7)	16	4.4	3.6
Groups (1) and (8)	40	5.5	7.3
Groups (1) and (9)	3	3.5	0.9
Groups (1) and (10)	7	3.6	1.9
Groups (1) and (11)	27	8.8	3.1
Groups (1) and (12)	27	3.3	8.2
Groups (1) and (13)	21	4.2	5.0
Groups (1) and (14)	29	4.6	6.3
Groups (1) and (15)	6	3.3	1.8
Groups (1) and (16)	1	3.0	0.3
Groups (1) and (17)	20	4.5	4.4
Groups (1) and (20)	28	4.8	5.8
Groups (1) and (21)	7	3.0	2.3
Groups (1) and (22)	11	4.2	2.6
Groups (1) and (23)	7	3.0	2.3
Groups (1) and (24)	16	3.7	4.3
Groups (1) and (25)	25	4.4	5.7

The base fat fed was a commercially hydrogenated vegetable shortening (fat A)[3] which was chosen because of its uniform composition and stability. Data showing the percentages of fat absorbed in 4 hours by adult rats fed 1.5 cc. of the fat followed by 1 cc. of a solution of the various substances tested are given in table 1.

Table 2 records the statistical significance of the differences in the means of the several groups.

[3] Fat A was a commercial product manufactured by hydrogenating cottonseed oil to an iodine number of approximately 70. The saponification number was 196.0.

DISCUSSION

Bile salts. Verzar and Kuthy ('29 b) have advanced the theory that the bile acids aid fat absorption by combining with the fatty acids, 'carrying' them through the intestinal wall. In a more recent publication Verzar and Laszt ('34) report an experiment in which they anesthetized rats with urethane, ligated a loop of the intestine—leaving the circulation intact, and after washing out the loop with physiological saline, injected into it a mixture of oleic acid and bile acid. After a definite time interval the amount of fatty acid absorbed was determined. They found that the absorption of oleic acid was definitely increased by glycocholic acid under the conditions of their experiment. In our experiments the addition of 1 or 50 mg. of commercial bile salts[4] did not increase the rate of absorption of fats. The addition of 500 mg. decreased absorption, although this figure (19%, table 1) is not a reliable one due to the laxative effect of this quantity of bile salts. No indications of diarrhea were observed in our animals when 1 or 50 mg. of bile salts were fed. In Verzar's experiment normal secretions into the intestinal tract were in part excluded; in ours, the digestive tract remained intact during the absorption period with the secretions and movements of the intestine functioning normally. Possibly in our experiments the optimum amount of bile acids was secreted by the liver, and, therefore, the further addition of small amounts of bile salts had no effect.

Sodium benzoate. Sodium benzoate is one of the many organic salts which shows hydrotropic properties. When fed with fat, 1 mg. did not affect the rate of absorption, but 50 or 500 mg. definitely lowered it (table 1).

Alcohol. Cori, Villiaume and Cori ('30) observed that small quantities of ethyl alcohol did not alter the rate of absorption of sugar. We obtained similar results with fat; a small quantity of alcohol had no marked effect on fat absorption, although 1 cc. of a 40% alcohol solution decreased it from 54% to 27% (table 1).

[4] Supplied through the kindness of the Wilson Laboratories. The preparation consisted predominately of crude sodium glycocholate and sodium taurocholate.

Peptone and sucrose. Since, in the ordinary diet, fat is usually absorbed in the presence of protein and carbohydrate, fat was fed with peptone, with sucrose, and with a mixture of the two. From the data presented in table 1 it is clear that fat was absorbed less rapidly when fed with these foodstuffs than when fed alone.

Calcium and potassium. The effect of electrolytes on absorption is, as yet, an unsettled question. Magee and Reid ('27), Magee and Southgate ('29), and Magee and Sen ('31, '32) have observed that small amounts of $CaCl_2$ in the lumen of the rabbit's intestine increased its tone and stimulated contraction. Glucose diffused more rapidly from sections of the surviving intestine into a Tyrode solution containing calcium than it did into a calcium-free solution. Magee and co-workers found also that thyroparathyroidectomized rats with a low blood calcium did not absorb as much glucose as normal rats and that the addition of calcium to the sugar solution increased absorption.

Contrary evidence has been presented by Gellhorn and Skupa ('33) who perfused the gut of frogs with a glucose solution and the blood vessels with a modified Ringer's solution. By the addition or omission of one salt or another these investigators found that when both calcium and potassium were omitted from the perfusiate, permeability of the gut to glucose was increased. The addition of $CaCl_2$ alone to such a solution decreased permeability, but when as little as 0.0075% of KCl was added, the permeability of the gut to glucose was increased.

Whether or not the action of calcium and potassium ions is specific for glucose absorption or is fundamental to the process as a whole cannot be determined until more evidence is presented. Under the conditions of our experiment small quantities of $CaCl_2$ or KCl had no effect on fat absorption; larger amounts of either of these salts depressed absorption (table 1).

Phosphates. Verzar and Laszt ('34) obtained a definite increase in the absorption of oleic acid when glycerophosphate was injected into ligated loops of rat's intestine along with oleic acid and bile acids. Sinclair ('29), Verzar and Laszt ('34),

and also Süllmann and Wilbrandt ('34) believe that the transformation of absorbed fatty acids into phospholipids is an essential step in the resynthesis of neutral fat in the intestinal mucosa. Additional evidence supporting this idea is to be found in the work of Smedley MacLean and Hoffert ('23) who found that yeast grown in an oxygenated medium containing phosphates stored much more fat than when grown on a phosphate-free medium. Magee and Reid ('31) reported that the presence of 0.2% NaH_2PO_4 in a 0.75 M glucose solution increased its rate of absorption by 50%. This work has been confirmed by Wilbrandt and Laszt ('33), and it has been suggested that the formation of glycerophosphoric acid is a step in the absorption of glucose. In our experiments NaH_2PO_4, glycerol, and sodium glycerophosphate solutions were fed along with fat, and the absorption of the fat determined. In table 1 it can be seen that the presence of any one of these substances in the intestinal tract during digestion and absorption of fat retarded rather than stimulated the process.

SUMMARY

The influence of water, bile salts, sodium benzoate, ethyl alcohol, peptone, sucrose, KCl, $CaCl_2$, NaH_2PO_4, glycerol and sodium glycerophosphate on the rate of absorption of fats has been determined. In general the feeding of small amounts of any one of these substances had little or no effect, but larger amounts invariably decreased the rate of fat absorption.

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RELATION OF CALCIUM AND OF IRON TO THE ERYTHROCYTE AND HEMOGLOBIN CONTENT OF THE BLOOD OF RATS CONSUMING A MINERAL DEFICIENT RATION [1]

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TWO FIGURES

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The fact that extreme restriction of the inorganic constituents of the diet produces significant alterations in the concentration of pigment and of erythrocytes in the blood of the rat has been repeatedly demonstrated (Smith and Schultz, '30; Swanson and Smith, '32; Orten and Smith, '34 a). There occurs a progressive increase in the number of erythrocytes per unit volume of blood and a simultaneous decrease in the concentration of hemoglobin. Careful analyses of the diet employed (Smith and Smith, '34) have shown that it is extremely deficient in calcium and, to a lesser extent, in potassium, sodium, phosphorus, magnesium, and chloride. Likewise, the amount of iron present in the low-salt ration (Brooke, '33) is somewhat less than that commonly believed to be required by the normal albino rat.

There arises the question of the nature of the specific inorganic deficiency or deficiencies responsible for the polycythemia and concomitant anemia observed in the low-salt animals. Preliminary studies demonstrated that the blood

[1] A preliminary report was made before the meeting of the Society of Biological Chemists in New York, March, 1934. Aided by a grant-in-aid from the National Research Council, 1934.

[2] National Research Council Fellow, Yale University, 1933–1934. Alexander Brown Coxe Fellow, Yale University, 1934–1935.

[3] Deceased.

changes were due solely to a lack of inorganic elements since the administration of the complete Osborne and Mendel salt mixture to the polycythemic rats restored the levels of pigment and of erythrocytes to normal. In view of the well-known relation of iron to hemoglobin formation, an involvement of this metal in the low-salt hematological syndrome was early suspected, even though it was realized that an iron deficiency usually produces a decrease in the erythrocyte content of the blood as well as a decrease in the concentration of pigment. The extreme deficiency of calcium in the experimental ration and the suggested relation (von Wendt, '05; Sherman, '07) of this element to the economy of the metabolism of iron also indicated a possible relation of calcium to the observed hematological abnormalities. For these reasons, the effect of the administration of iron and of calcium on the blood of rats consuming a mineral deficient ration was investigated.

EXPERIMENTAL

Male albino rats of the Connecticut Agricultural Experiment Station strain, weighing from 40 to 50 gm. at weaning (21 days of age), were employed as experimental animals. They were housed in individual wire cages having a wide mesh ($\frac{1}{2}$ inch) bottom designed to minimize the opportunity for coprophagy. The weanling animals were fed the stock colony ration (Orten and Smith, '34 b) until they attained a weight of 120 ± 4 gm. In order to further insure uniform, vigorous experimental subjects, only those animals which attained the above weight at 35 ± 2 days of age were used. These rats were then subjected to two general types of experimental procedures, the 'curative' and the 'preventive,' described below.

Curative procedure. For a preliminary period of 8 weeks, the 35-day-old animals were fed a basal 'low-salt' ration having the following composition: Specially prepared low-ash casein, 18%; dextrin, 55%; hydrogenated vegetable oil,[4]

[4] Crisco.

27%. The daily vitamin supplements consisted of wheat germ extract, 1 cc.; dried yeast, 200 mg.; cod liver oil, 100 mg. and wheat germ oil, 40 mg. The source of the various dietary constituents and the methods of preparation have been described elsewhere (Swanson and Smith, '32; Swanson, '30). At the end of the 8-week preliminary period, the various mineral supplements to be studied were added to the ration and the animals were observed for a further 10-week experimental period. Calcium was fed as 'C.P.' calcium carbonate at a level of 50 mg. calcium daily, the amount ingested daily in the form of the salt mixture (Osborne and Mendel, '17) by comparable normal control animals. This quantity of calcium should be sufficient to satisfy the daily requirement of the adult rat (Osborne and Mendel, unpublished data; Whitcher, Booher and Sherman, '33), even during pregnancy and lactation (Cox and Imboden, '36). Iron was fed as purified, copper-free ferric chloride[5] at a level of 1.4 mg. daily, the amount consumed daily by the normal control rats. Three groups of control animals were studied. One of these was continued on the basal low-salt ration; another was given the complete salt mixture as a supplement, at a level of 408 mg. daily, the average amount ingested by normal control animals; and the third group was given chloride at a level of 2.34 mg. HCl daily (N/10 solution), as a control to the ferric chloride-supplemented group. In all cases, the mineral supplements were mixed directly in the basal ration which was fed daily in an amount equal to the average daily quantity consumed by the animal during the final 2 weeks of the preliminary period. The reason for the feeding of this 'restricted' allowance of the basal ration was to prevent possible changes in the amount of basal ration consumed after supplementation, a variation which would have rendered difficult an interpretation of subsequent changes in the composition of the blood in terms of the inorganic supplement per se. Normal control animals

[5] Appreciation is expressed to Dr. W. W. Watson of the Department of Physics for a spectrographic examination of this sample of ferric chloride.

were maintained during the entire preliminary and experimental periods. These received an ad libitum allowance of the basal low-salt ration, in which 4% salt mixture replaced an equal amount of dextrin, and the same amounts of vitamin supplements as the experimental animals.

Preventive procedure. The young rats, 35 days old, were changed directly from the stock colony ration to the mineral-supplemented low-salt ration and observed for a 10-week period. Calcium, as the 'C.P.' or specially purified, iron-free carbonate, and iron, as the purified chloride or the citrate, were fed in the same daily amounts described above. Control animals were fed either the unsupplemented low-salt ration, or the ration supplemented with hydrochloric acid, or with the entire salt mixture. In all instances, the inorganic adjuvants were incorporated directly in the basal ration. In order to keep the mineral supplement as the sole controllable variable in the experiment, all members of the various groups received the same 'restricted' amount of food daily. This quantity was calculated from previous food consumption records[6] of 105 rats receiving the same basal diet as that employed in the present work. The daily allowance of the basal ration, adjusted weekly to that of the recorded averages, was as follows: First week, 7.1 gm.; second week, 6.4 gm.; third to fifth weeks, 5.7 gm.; sixth to tenth weeks, 5.6 gm.

The body weights of the various animals were recorded weekly and the hemoglobin content and erythrocyte count of the blood were determined bi-weekly during the entire period of observation. The methods employed for the blood determinations have been described elsewhere (Orten and Smith, '34 b).

RESULTS

The averaged data obtained in this investigation, with the exception of the body weight values, are given as composite curves in the accompanying charts Some of the body

[6] Appreciation is expressed to Dr. Miriam F. Clarke and Dr. Paul K. Smith for making available the food intake data collected on their rats fed the low-salt ration.

weight findings may be described briefly. The normal control animals used in the 'curative' study grew at a rate similar to that of comparable stock rats (Orten and Smith, '34 b) while those consuming the low-salt ration showed a typical retardation of growth (Swanson and Smith, '32), reaching and maintaining an average maximum body weight of approximately 160 gm. The animals given the supplement of the complete salt mixture at the end of the preliminary 8-week period increased on the average approximately 40 gm. in weight during the 10-week realimentation period while those given only calcium gained some 20 gm. The animals receiving ferric chloride, however, progressively lost weight during the entire realimentation period. Similar results were obtained in the animals studied by the 'preventive' procedure. At the end of the 10-week experimental period, the groups given either the complete salt mixture or calcium had increased approximately 20 gm. more in body weight than the low-salt controls, whereas those given ferric chloride (or HCl) weighed slightly less than the unsupplemented controls. It should be borne in mind that the growth effects described are due solely to the influence of the various inorganic supplements since the allowance of energy-yielding basal ration in all cases was 'restricted,' and therefore did not vary between groups.

The averaged erythrocyte and hemoglobin data obtained on the groups of rats studied by the 'curative' procedure are given in figure 1. The erythrocyte counts and pigment concentrations of the blood of the normal control animals fed the adequate ration showed the characteristic increase with age (Orten and Smith, '34 b) and attained adult levels after some 6 weeks on the experiment. The animals fed the low-salt ration, however, developed the typical polycythemia and mild anemia. These abnormal values persisted in the animals continued on the low-salt ration, although there was a tendency for the average erythrocyte count of the group to decrease because of a marked premortal fall in the value in certain members which succumbed during the experiment. The addition of the complete salt mixture to the low-salt diet at the

end of the 8-week preliminary period was followed by a prompt, progressive decrease in the erythrocyte count and an increase in pigment concentration to normal values. Similar

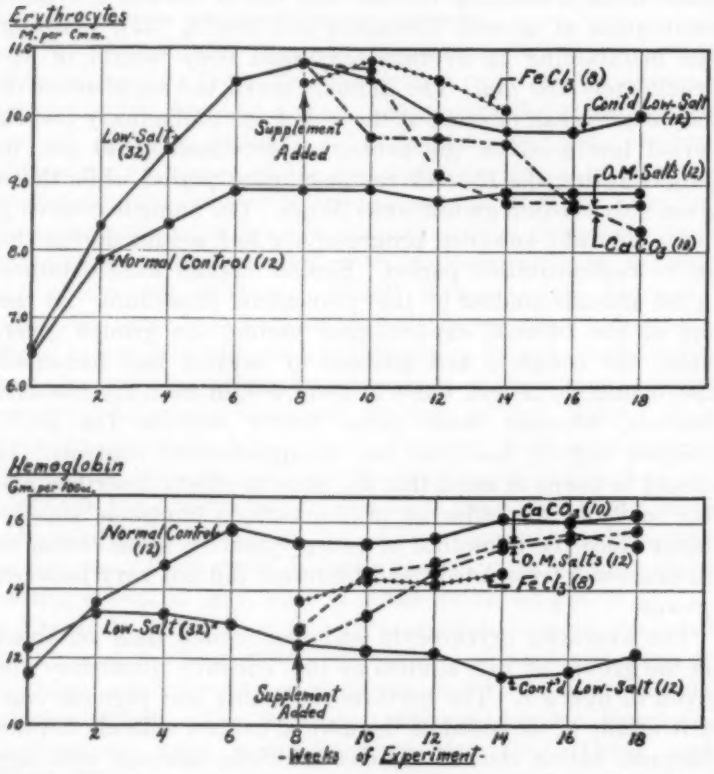

Fig. 1 'Curative procedure.' Average erythrocyte and hemoglobin content of the blood of control rats and of rats consuming a 'low-salt' ration supplemented by certain inorganic elements. The figures in parentheses indicate the numbers of rats in the groups.

results were obtained with calcium; without exception, there was a restoration of the cell count and hemoglobin level to values within the normal range. There was a similar tendency in some of the animals fed ferric chloride. However,

the results were not convincing because the responses to iron were exceedingly variable, some animals showing a decrease in erythrocytes and a rise in hemoglobin, others showing

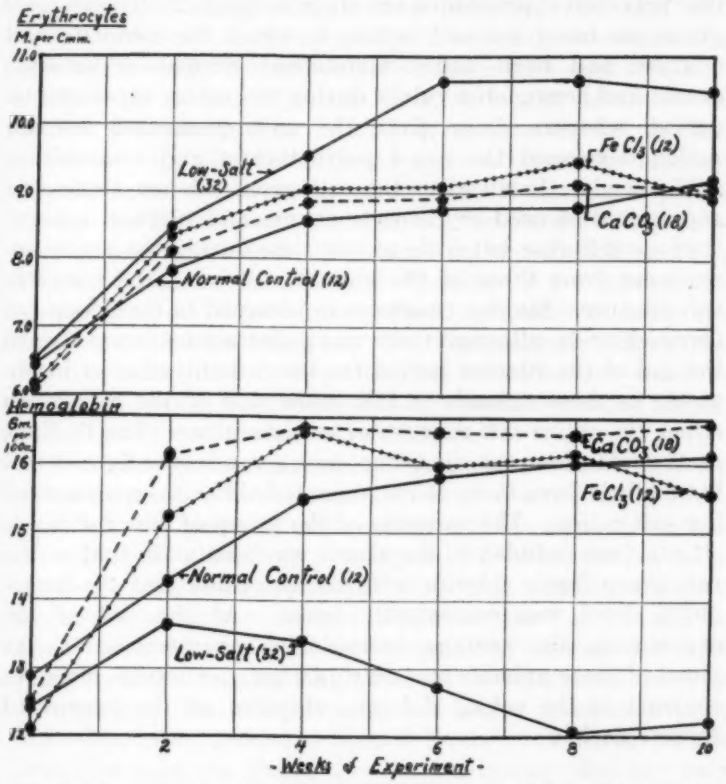

Fig. 2 'Preventive procedure.' Average erythrocyte and hemoglobin content of the blood of control rats and of rats consuming a 'low-salt' ration supplemented by certain inorganic elements. The figures in parentheses on the various curves indicate the numbers of rats in the groups.

either no change or a slight aggravation of the condition, and because the majority of the animals died after a few weeks, some within a few days, of supplementation. The findings in animals fed hydrochloric acid (not included in the chart) did

not differ significantly from those in the animals continued on the basal ration alone.

The averaged blood data for the groups of rats studied by the 'preventive' procedure are given in figure 2. The animals given the basal low-salt ration to which the complete salt mixture had been added maintained normal erythrocyte counts and hemoglobin values during the entire experimental period, whereas those given the unsupplemented low-salt ration developed the usual polycythemia and concomitant mild anemia. In all cases the rats given calcium alone as a supplement showed erythrocyte counts and pigment concentrations differing but little at any time during the entire experiment from those of the animals receiving the complete salt mixture. Similar results were obtained in the group fed ferric chloride, although there was a distinct tendency toward the end of the 10-week period for the concentration of hemoglobin in these animals to fall below that of the rats given either the entire salt mixture or calcium alone. The findings on the 'chloride control' group (not given in the figure) differred little from those of the animals fed the unsupplemented low-salt ration. The response of the group of rats fed ferric citrate (not included in the chart) was similar to that of the rats given ferric chloride with the exception that the hemoglobin level was consistently lower. At the end of the experiment, the average hemoglobin concentration of the blood of these animals was only 14.0 gm. per 100 cc. blood, in contrast to the value, 15.4 gm., obtained on the group fed ferric chloride.

DISCUSSION

From the results obtained with the groups of animals receiving the complete salt mixture, it is evident that the polycythemia and concurrent mild anemia observed in rats fed a diet deficient in inorganic salts are due exclusively to the low mineral intake. Furthermore, the fact that purified calcium alone produces a similar effect in the restoration or maintenance of normal erythrocyte and hemoglobin values indicates that a lack of this element is intimately involved in the

hematological abnormalities observed. Likewise, the response obtained with iron in the 'preventive' study points to an involvement of this element. Additional experiments (unpublished observations) have indicated that the other common dietary inorganic elements, sodium, potassium, magnesium, and chloride, are not concerned in this condition, since neither the adding of these elements to the basal low-salt ration nor the feeding of a salt mixture devoid of them produces any alteration in the results otherwise obtained. However, the fact that the substitution of edestin, a protein containing little, if any, phosphorus, for the phosphoprotein, casein, results in less drastic changes in the composition of the blood (Swanson, Timson and Frazier, '35) suggests that phosphorus, or perhaps the calcium to phosphorus ratio, may be involved in this condition. The foregoing results warrant the conclusion that the hematological abnormalities occurring in rats fed the diet deficient in inorganic salts are due largely, if not entirely, to the lack of calcium and/or iron.

There are certain suggestions in the literature which may serve as a basis for an explanation of the favorable effects obtained with iron and with calcium in this study. It has been observed (Eveleth, Bing and Myers, '33) that rats fed a diet of whole cow's milk, apparently containing a sufficient amount of copper, supplemented by a minimal amount of iron did not show the usual iron-deficiency anemia but developed a strikingly polycythemia accompanied by a subnormal level of hemoglobin. The administration of larger doses of iron, on the other hand, produced normal levels of erythrocytes and hemoglobin in the blood, thus demonstrating that the polycythemia-anemia was due entirely to a lack of a sufficient amount of iron. This hematological syndrome and its relation to iron is remarkably similar to that observed in the animals consuming the low-salt ration. It is possible that in both instances the polycythemia is merely a compensatory response, that is, a production of increased numbers of smaller-sized erythrocytes to compensate for a subnormal level of pigment due to the availability of only a minimal amount of

iron. If this is true in the case of the animals fed the mineral deficient ration, the beneficial action of iron may simply be a correction of a slight iron deficiency, thus promoting the maintenance of a normal concentration of hemoglobin in the blood and, in turn, a normal number of erythrocytes.

The failure of iron to exert a consistently favorable effect when administered as a curative agent merits some comment. The fact that a definite rise in the level of hemoglobin and a simultaneous decrease in the erythrocyte count occurred in some animals whereas little or no change or even a decrease in the concentration of pigment was observed in others may be interpreted as indicating that the response to iron was dependent upon the presence of some other substance. In view of the invariably favorable responses to calcium supplementation, it appears likely that the other substance is calcium itself and that the inconsistent results obtained with iron in the curative procedure may have been due to variations in the amounts of reserve calcium in the different animals. Likewise, the favorable effect of iron in the preventive study may also have been dependent upon the presence of reserve calcium in the young animals before depletion on the salt-poor, basal ration. Even in these animals, however, there was a distinct tendency toward the end of the 10-week period for the concentration of hemoglobin to decrease below that in the blood of rats given either the complete salt mixture or calcium alone.

The consistently favorable results obtained with calcium are perhaps more difficult to explain. Although there is some evidence which appears to indicate the contrary (Shelling and Josephs, '34; Kletzien, '35), it is possible that under certain conditions this element exerts a beneficial effect on the economy of iron in metabolism, as was suggested by the results of a limited number of balance studies in human subjects (von Wendt, '05; Sherman, '07). The recent finding (Ramage, Sheldon and Sheldon, '33) that there is a remarkable degree of correlation between the amount of calcium and that of iron present in the livers of human infants may be interpreted as further evidence in support of this view.

It is possible that under the conditions of the present experiment calcium added to the diet increases the absorption or utilization of iron and therefore that the favorable results obtained with this element are simply an indirect iron effect. Some support to the suggestion of an impairment of iron metabolism in the low-salt animals is given by the fact that a favorable hemoglobin response was not consistently elicited by iron, particularly when fed as the citrate, and even by ferric chloride toward the end of the preventive experiment. In this connection, the statement of one investigator (Davidson et al., '33) regarding the relation between calcium, iron, and anemia is of interest: ". . . . a normal blood level can be maintained on a diet rich in calcium and moderate in iron but that an anemia will occur on the same iron intake if the calcium is reduced." Thus, the possibility that calcium exerts an 'iron-sparing action' under the conditions of the present experiment, must be considered in interpreting the results herein presented.

A number of instances are found in the clinical literature which also indicate a possible relationship between calcium, or some factor controlling its metabolism, and the amount of hemoglobin in the blood. For example, in proved cases of hyperparathyroidism, there is often an anemia (Albright, Aub and Bauer, '34) due, according to the authors, to the replacement of some of the marrow cavity with fibrous tissue. An explanation on a chemical basis would seem to be equally tenable. Likewise, in a case of acromegaly with osteoporosis (Scriver and Bryan, '35) an anemia was recorded. Other similar examples are found scattered widely through the clincal literature.

Thus, there is a considerable amount of evidence both from studies on experimental animals and on man indicating a possible relationship between calcium, or some factor controlling its metabolism, iron, and the concentration of hemoglobin in the blood. The data obtained in the present study appear to support such a view since, under the conditions employed, rats receiving the mineral deficient ration develop a

chronic anemia with an accompanying microcytic polycythemia whereas other animals consuming the same amount of the same diet under identical experimental conditions but given calcium maintain a normal concentration of pigment and erythrocytes in the blood.

SUMMARY

The administration of a complete salt mixture to rats fed a basal ration extremely low in inorganic salts both alleviates and prevents the marked polycythemia and concurrent mild anemia which otherwise persist or develop.

Calcium alone, fed as the 'C.P.' or highly purified carbonate, exerts a comparable restorative and preventive action.

Iron alone, fed as purified ferric chloride, shows a similar preventive effect but is not consistently efficacious as a restorative agent.

Further available data indicate that none of the other common inorganic elements known to be deficient in the salt-poor diet, with the possible exception of phosphorus, are concerned in the production of the blood changes under discussion.

It appears, therefore, that the hematological abnormalities which occur in rats as a result of the feeding of the mineral deficient ration are due chiefly, if not entirely, to a lack of calcium and/or iron.

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THE INVERSE RELATION BETWEEN GROWTH AND INCIDENCE OF CATARACT IN RATS GIVEN GRADED AMOUNTS OF VITAMIN G-CONTAINING FOODS [1]

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ONE FIGURE

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In 1931 a paper from this laboratory (Day, Langston and O'Brien, '31) reported the consistent occurrence of cataract in rats given a diet deficient in vitamin G. More recently (Day and Langston, '34) we reported a larger series of animals, and emphasized the probable significance of cataract as an evidence of G avitaminosis. O'Brien ('32) and Yudkin ('33) likewise found cataract resulting from vitamin G deprivation in a large percentage of animals. Bourne and Pyke ('35), however, obtained only 31% cataract as a maximum in any group of rats. They used a diet essentially the same as ours, but started with much larger animals than we usually employ in vitamin G experiments.

For several years we have been engaged in the assay of various foods for vitamin G. In addition to keeping weekly weight and food consumption records, we have made routine weekly examinations of the eyes of all rats with an ophthalmoscope. The investigator making the eye examinations has always been impressed by a striking relationship between

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failure of growth and appearance of cataract. Negative controls and rats receiving supplements containing very little vitamin fail to grow, and develop an unkempt appearance of the hair. As the experiment progresses such rats exhibit keratitis, and, almost invariably, cataract. Litter mates receiving foods that contain the vitamin grow, and usually fail to develop cataract. It has always been observed that the greater the growth, the less is the probability that cataract will develop. In handling such experimental animals over a period of several years we have become convinced that there must be some fundamental inverse relationship between growth and development of cataract. Furthermore, we have employed a wide variety of natural food substances, and extracts of yeast and liver, and we have never found a substance that would consistently promote growth and fail to prevent cataract, or one that would prevent cataract and fail to promote growth. That is, the growth-promoting and cataract-preventive properties of substances always appear to run parallel, which would lead one to suspect that these properties may reside in the same chemical compound. Such observations are, by their nature, not readily amenable to quantitative expression. We have attempted, however, to tabulate some of our data in such a way as to give a quantitative statement regarding the correlation between growth and incidence of cataract in our experimental animals.

EXPERIMENTAL METHOD

The composition of the diet used in this laboratory for the assay of what we shall call vitamin G has been described elsewhere in detail (Day and Langston, '34) and need not be repeated here in its entirety. Young from litters of eight to twelve rats each are placed on experiment when they are 21 days of age, at which time they weigh from 30 to 45 gm. They are given diet 625 in non-scatter food cups, and distilled water ad libitum. The diet is made up exactly as described in the previous publication. Since the source and treatment of the casein used may have an effect upon the development

of cataract, a statement should be made regarding the kind we use. We have used 'casein, edible, muriatic,'[2] extracted with 60% alcohol in the laboratory according to the method of Sherman and Spohn ('23). In some experiments we have employed 'vitamin-free' casein.[3] The animals are housed in cages with raised metal floors of $\frac{3}{8}$ inch mesh, and are maintained at a nearly constant temperature.

After 2 weeks on the basal diet alone, each animal is given a pre-determined amount of a food substance, accurately weighed. This is usually fed either three or six times weekly, depending upon the nature and quantity of the material. One or more animals from each litter are retained as negative controls. The animals receiving addenda are killed at the end of 10 weeks (that is, after they have received the supplements 8 weeks), while the control animals are kept until death intervenes.

Using the foregoing procedure, we have made assays of a number of different substances during the past 2 years. The list of materials fed includes several kinds of cheese, liver extracts, yeast, lamb, pork, pork liver, ham, bacon and other materials.

RESULTS AND DISCUSSION

If we may assume that the basal diet used furnishes an adequate amount of all necessary growth essentials except one, then the rate of growth of experimental animals receiving such diet should be proportional to the amount of this one substance made available to them. The growth of any animal, then, should be a measure of all the vitamin to which it has access: any reserve bodily store, plus any trace of vitamin in the basal diet, plus any obtained by access to excreta or from other adventitious sources, plus any fed to the animal in a food supplement. In our experiments we have attempted to eliminate, as far as possible, all of the variables except the food supplement. Uniform cages and feeding vessels are

[2] Manufactured by the Casein Company.
[3] Casein Company's Labco brand.

employed, meticulous care is exercised to prevent contamination of the basal diet or cages with vitamin-bearing substances; access to excreta is prevented as far as possible; diet constituents are obtained from constant sources in large quantities, and are given uniform treatment in preparation in this laboratory. All the animals used come from one stock that has received a uniform diet for several generations; only animals from large, healthy, uniform litters are employed in experiments, and they are started on experiment at a definite age when they fall within certain weight limits. With all these precautions, however, there is observed some variation between animals within a single litter, and frequently considerable variation between animals of different litters. Since such differences are frequently manifest in parallel experiments, it is believed that the chief uncontrolled variable in our experiments is the pre-experimental body store of vitamin.

The data from all vitamin G assays over a period of more than a year have been collected and tabulated. All the animals receiving the basal diet only (controls) have been grouped together. The animals receiving vitamin supplements have been grouped according to their growth during the 10-week experiment. Those making net gains in 10 weeks of 20 gm. or less have been placed in group I, those making net gains of 21 to 40 gm. in group II, those making gains of 41 to 60 gm. in group III, and those gaining more than 60 gm. in group IV. It must be admitted that this grouping is quite arbitrary, and that some other division of the animals very probably would have served the purpose just as well.

As an example of the type of supplements fed the animals, one illustration will serve. A series of thirty rats was used in an assay of pork liver. The growth of seven rats receiving 0.05 gm. fresh liver daily placed them in group I. Three rats receiving 0.05 gm. and nine rats receiving 0.1 gm. daily fell in group II. One rat receiving 0.1 gm. daily and three rats receiving 0.2 gm. daily were placed in group III since the total net growth of each in 10 weeks was between 41 and 60 gm. Seven rats receiving 0.2 gm. liver grew more than 60 gm. during the 10 weeks, and so were placed in group IV.

A summary of the data is given in table 1. Of the seventy-eight controls included in this series, only four made net gains of more than 20 gm. during the 10 weeks, and only twenty-three made net gains of more than 10 gm. during the same period. Forty-nine (or 63%) of these control animals exhibited cataractous changes during the 10-week period, and a total of sixty-seven (86%) developed cataract before death. The incidence of cataract in the groups receiving supplements diminished with increasing growth; the four groups showed 39%, 26%, 14%, and 0% cataract, respectively. None of the

TABLE 1

Table showing incidence of cataract in rats receiving graded amounts of vitamin G-containing foods, grouped according to growth during a 10-week period

	GROWTH RANGE DURING 10-WEEK PERIOD	MEAN NET GROWTH IN 10 WEEKS	NUMBER OF RATS	CATARACT	
				Number	Incidence
	gm.	gm.			%
Controls	— 3 to 27	9	78	49	63[1]
Group I	Up to 20	14	36	14	39
Group II	21 to 40	28	62	16	26
Group III	41 to 60[2]	50[2]	28	4	14
Group IV	Above 60	80	28	0	0

[1] Although only 63% of control animals developed cataract in 10 weeks, 86% exhibited cataract before death.

[2] None of the rats growing more than 56 gm. during the 10-week period developed cataract.

animals that made net gains of more than 56 gm. during the period developed cataract, and of forty-seven animals making net gains of more than 30 gm., only seven exhibited cataract. It is thus apparent that cataract was only rarely found in animals receiving enough vitamin to promote growth in excess of an average of 3 gm. weekly.

Figure 1 presents the same data in graphic form, and clearly shows the influence of a very small amount of vitamin on the incidence of cataract. Although the animals in group I made an average gain of only 5 gm. more in 10 weeks than did the control animals, the incidence was much lower—39% as compared with 63% for controls.

The specific type of dermatitis referred to as 'florid' dermatitis by Chick, Copping and Edgar ('35) and photographically shown by Bender et al. ('36) was not observed in any of the animals receiving the diet used in these experiments, although it develops regularly at about 28 days in rats receiving crystalline vitamin B(B_1) in place of the rice polish extract. This would indicate that our 80% alcohol extract of rice polish contains a considerable amount of the rat dermatitis-preventive factor.

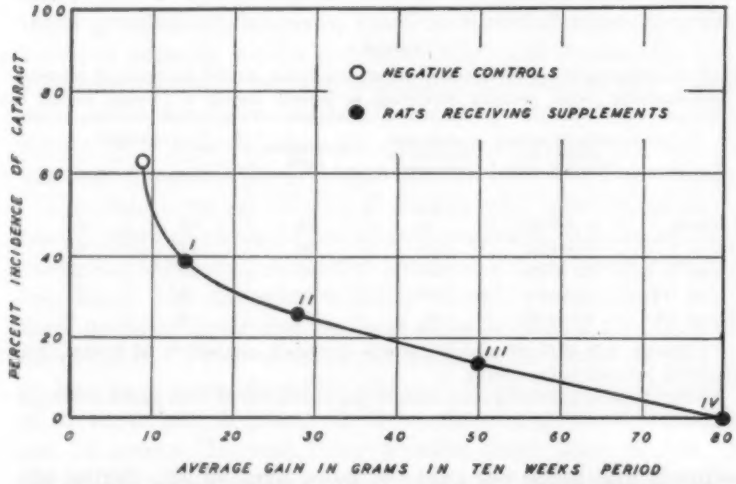

Fig. 1 Chart showing incidence of cataract in groups of rats plotted against average growth. The roman numerals refer to the groups as given in table 1.

Ulceration of the skin was observed occasionally in animals at all levels of growth, and was about as common in animals growing more than 60 gm. (group IV) as in controls or in rats of groups I and II. Roughness and loss of hair was observed in animals of all groups. Definite alopecia was seen in about 35% of the control group and of groups I and II. Loss of hair was less common in rats at the higher levels of growth. Keratitis was observed in practically all animals exhibiting cataract, and in many animals not showing cataract.

Its consistent accompaniment of cataract in these experimental animals leads us to believe that it is certainly a manifestation of the same dietary deficiency that predisposes to cataract.

We are now investigating the possibility of the identity of flavin with the cataract-preventive factor. It appears possible that flavin may have cataract-preventive properties, but we do not yet have conclusive proof that such is the case. However, what is learned in the future regarding the identity or non-identity of flavin and the cataract-preventive substance may slightly alter the interpretation of the results herein reported, but we believe will not detract from their significance.

SUMMARY

More than 200 young rats, weighing between 30 and 45 gm., were used in a series of vitamin G assays. For the first 2 weeks they were given a deficient diet only, after which time they were given daily supplements of vitamin-containing foods. Control animals receiving no supplement were kept until death, whereas the animals receiving supplements were killed after 10 weeks.

Eighty-six per cent of seventy-eight controls developed cataract before death, although only 63% showed such eye changes during the 10-week period. The rats receiving vitamin supplement, grouped according to growth during the 10-week period, showed the following incidence of cataract: animals growing 20 gm. or less—39% cataract; animals gaining from 21 to 40 gm.—26% cataract; animals gaining from 41 to 60 gm. —14% cataract; animals gaining more than 60 gm.—0% cataract. It is thus apparent that there was an inverse relationship between growth and appearance of cataract; that is, the greater the growth, the lower the incidence of cataract. This would seem to indicate that, under the conditions of the experiment, growth was a measure of the cataract-preventive property of the supplement. These data also indicate that only a small amount of the vitamin is required to prevent the appearance of cataract.

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BLOOD SUGAR IN RATS RENDERED CATARACTOUS BY DIETARY PROCEDURES [1]

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ONE FIGURE

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Within the past 5 years it has been shown that cataract may be produced in rats by either of two different nutritional methods. A series of papers from this laboratory (Day, Langston and O'Brien, '31; Langston and Day, '33; Day and Langston, '34) described the consistent occurrence of cataract in albino and Norway rats which had been given a diet deficient in one of the relatively heat-stable factors of the vitamin B complex (vitamin B_2 or G). O'Brien ('32), Yudkin ('33) and Bourne and Pyke ('35) published data corroborating, in part at least, the results obtained in this laboratory.

Mitchell and Dodge ('34, '35) reported the occurrence of cataract in rats given diets containing high percentages of lactose, and later it was shown that galactose produced cataract in a much shorter period (Mitchell, '35; Yudkin and Arnold, '35). It is thus evident that cataract may be produced readily in rats, on the one hand by a deficiency of vitamin G or some closely associated factor, and on the other hand by the presence of large percentages of lactose or galactose in the diet.

It seemed quite possible to the author that the cataract resulting from lactose or galactose feeding might be the manifestation of an abnormal carbohydrate utilization or metabolism. Consequently, the experiments of Mitchell and Dodge

[1] Research paper no. 503, journal series, University of Arkansas.

were repeated, and blood sugar determinations were made upon the animals receiving lactose as well as upon control animals. Preliminary determinations indicated that the lactose rats had elevated blood sugar levels. Experiments were therefore planned to show whether, under carefully controlled conditions, animals receiving high percentages of galactose and lactose would exhibit significant hyperglycemia when compared with animals receiving other common glycogen-forming carbohydrates. Although there was very little reason to believe that an abnormal carbohydrate metabolism would likewise be associated with vitamin G deficiency, it was felt that parallel determinations of blood sugar upon animals developing cataract as a result of vitamin deprivation should be of interest.

EXPERIMENTAL METHODS

Animals receiving normal diets containing various carbohydrates. Young albino rats from our own stock, weighing 30 to 45 gm., were placed in individual metal cages and fed normal diets containing various utilizable carbohydrates. The composition of the diets was as follows:

	Per cent
Casein, commercial edible	18
Salt mixture, Osborne and Mendel ('19)	4
Cod liver oil[1]	2
Butter fat	6
Yeast, dried[2]	10
Carbohydrate	60

The carbohydrates used were glucose, galactose, sucrose, lactose and starch. The animals in each litter were divided, so far as possible, between the diets containing the different carbohydrates. Weight, food intake, and ophthalmoscopic records were made at weekly intervals.

Blood sugar determinations were made upon blood taken from the tails of the animals at frequent intervals. Sugar was determined by a slight modification of the Folin micro method ('28). Since it was found difficult to obtain even 0.1 cc.

[1] Supplied by the E. L. Patch Company, Boston.
[2] Supplied by the Fleischmann Laboratories, New York.

of blood from the tails of some of the animals, special pipettes calibrated to contain 0.025 cc. were made for the purpose. The blood was collected in these pipettes and diluted with the tungstic acid solution to 2.5 cc. After centrifuging, 2 cc. of the clear filtrate was used for the colorimetric determination, and the final dilution was made in a tube calibrated at 12.5 cc. Only half the usual quantities of reagents were employed; otherwise the technic as described by Folin was followed. Except when diet was withheld for the purpose of making tolerance tests, the rats had access to food at all times. Since young rats eat at frequent intervals, it may be assumed that most of the determinations were made during the absorption of food from the digestive tract.

No attempt was made to determine, qualitatively or quantitatively, the nature of the sugar in the blood. It is assumed, however, that at least a part of the sugar in the blood of rats receiving lactose was galactose, and probably the extremely high values resulting from galactose feeding were due, in part, to the presence of this sugar in the blood. Since galactose is no more strongly reducing toward the reagent used in the Folin micro sugar method than is glucose, the results obtained and here reported should be fairly accurate estimates of the total sugar content of the blood.

On several occasions carbohydrate tolerance tests were run upon groups of the animals, using representative litter mates receiving different carbohydrates. In these tests the diet was removed in the afternoon or evening, and sugar determinations were made early the following morning, after which the rats were given access to a weighed quantity of diet for a period of ½ hour or 1 hour only. Sugar determinations were then made at hourly intervals during the day. The uneaten residue from each animal's food was weighed to make certain that differences in blood sugar curves were not due to differences in amount of food ingested.

Vitamin deficient animals. Young rats were given the vitamin G-deficient diet (no. 625) which has previously been described in detail (Day and Langston, '34). Under the conditions of heredity, age and size of animals, caging, etc., employed

in this laboratory, more than 75% of such animals developed cataract. Litter mates receiving the deficient diet supplemented with dried yeast (as 20% of the diet, replacing cornstarch) were used as normal controls. The experimental and control animals were given access to diet and distilled water at all times, and routine weight, food intake, and ophthalmoscopic records were kept. Blood sugar determinations were made upon deficient and normal animals by tail bleedings, using the Folin method modified as described above. In a few cases 'tolerance' tests were made.

RESULTS AND DISCUSSION

Seventeen out of the eighteen rats which were given lactose developed ophthalmoscopic cataract; the average time of appearance was 44 days. In only two cases did the cataracts

TABLE 1

Incidence of cataract in rats receiving an adequate diet containing various carbohydrates as 60% of the diet

CARBOHYDRATE FED	NUMBER OF RATS	NUMBER SHOWING CATARACT	TIME OF APPEARANCE OF CATARACT
			days
Glucose	9	0	..
Galactose	3	3	11
Sucrose	11	0	..
Lactose	18	17	44
Starch	11	0	..

progress to maturity. The three rats given galactose exhibited ophthalmoscopic cataract on the eleventh day, and mature cataracts on the nineteenth day. This cataract resulting from lactose or galactose feeding was quite different in appearance from the cataract caused by avitaminosis, especially during the early stages of its development. None of the rats that received glucose, sucrose, or starch showed any lenticular changes. The number of animals and incidence of cataract for the various carbohydrates are listed in table 1.

Table 2 gives a summary of data on the blood sugar in rats receiving the various carbohydrates. Since none of the determinations that were made upon rats in the fasting state

have been included in this tabulation, it is believed that the differences were due to the effect of the absorption of food from the intestinal tract. It is evident from a perusal of the table that glucose and starch gave blood sugar levels not significantly different from each other. Rats given sucrose showed slightly elevated blood sugar levels; the average of 133 mg. per 100 cc. blood may be, from a statistical standpoint, significantly greater than the averages for glucose and starch. The blood sugar in rats receiving lactose was extremely variable, values being found all the way from the fasting level up to 234 mg. per 100 cc. The average of eighty-nine determinations was 160 mg. per 100 cc., with a probable error of 2.3 mg.

TABLE 2

Blood sugar in rats receiving normal diets containing different carbohydrates

CARBOHYDRATE IN THE DIET	NUMBER OF RATS	NUMBER OF DETERMINATIONS	MEAN BLOOD SUGAR WITH PROBABLE ERROR
			mg. per 100 cc.
Glucose	9	45	121 ± 1.9
Galactose	3	27	372 ± 14.0
Sucrose	11	52	133 ± 2.2
Lactose	18	89	160 ± 2.3
Starch	11	47	123 ± 1.8

The probable error of the difference between blood sugar levels of glucose and lactose rats was 3.0 mg., which with a difference of 39 mg., means that the difference is thirteen times the probable error of the difference. If statistical methods may be applied to such observations, the chances are more than 1,000,000,000 to 1 that the difference between mean blood sugar levels in the lactose and in the glucose rats was significant.

Because of the cost of the sugar only three rats were given the galactose-containing diet, but twenty-seven determinations were made upon these three rats. Blood sugars were found to be high and quite variable; one determination showed 556 mg. sugar per 100 cc. of blood. The average of the twenty-seven determinations was 372 mg. per 100 cc., with a probable error of 14 mg. Comparing the average sugar levels in the galactose and glucose animals, and their probable errors, it is

evident that the difference between the two is seventeen times the probable error of the difference. Again, if statistical methods may be applied to such observations, the chances are much more than 1,000,000,000 to 1 that the difference was significant.

A few determinations made upon the experimental animals in the fasting state seemed to show no difference between animals which had been receiving the different carbohydrates. That is, the fasting level of sugar in galactose and lactose rats was not significantly different from corresponding levels of sugar in litter mates receiving other carbohydrates. This might indicate that the lactose and galactose had not caused any permanent derangement in carbohydrate metabolism.

Figure 1 shows graphically the results of a 'tolerance' test, which is typical of those obtained. In this case the diet of all the animals to be used was removed at 4.30 P.M. At 8.30 A.M., after a 16-hour fast, blood was drawn for analysis, and a weighed quantity of diet was given to each rat. After the animals had had access to the diet for 1 hour it was removed, and blood was drawn for analysis at intervals during the day. Within an hour the sugar in the blood of the galactose rat increased from 125 mg. to 532 mg. per 100 cc. It is probable that the peak of the curve was not recorded. At the end of 2½ hours the blood sugar was falling, and it reached a normal level at the end of 7½ hours. On the other hand, blood sugar in the animal receiving lactose rose rather slowly, and was still rising at the end of 7½ hours, at which time it exhibited 200 mg. sugar per 100 cc. blood. The curves of blood sugar for the animals receiving sucrose and glucose were similar to that of the animal receiving starch, and have been omitted from the chart. Speculating on the significance of these curves, it seems apparent that the galactose, requiring no digestion, was absorbed rapidly from the intestinal tract, and yet was only slowly converted into and stored as glycogen. The lactose, on the other hand, required digestion, so that absorption was much slower and the peak of the blood sugar curve was reached much later.

Since the mechanisms operating to cause cataract are very poorly understood, any attempt to explain the production of cataract in these experimental animals in terms of blood sugar level would be largely conjecture. However, there are some comments that may not be altogether inappropriate in discussing the problem. It is interesting that the carbohydrate that

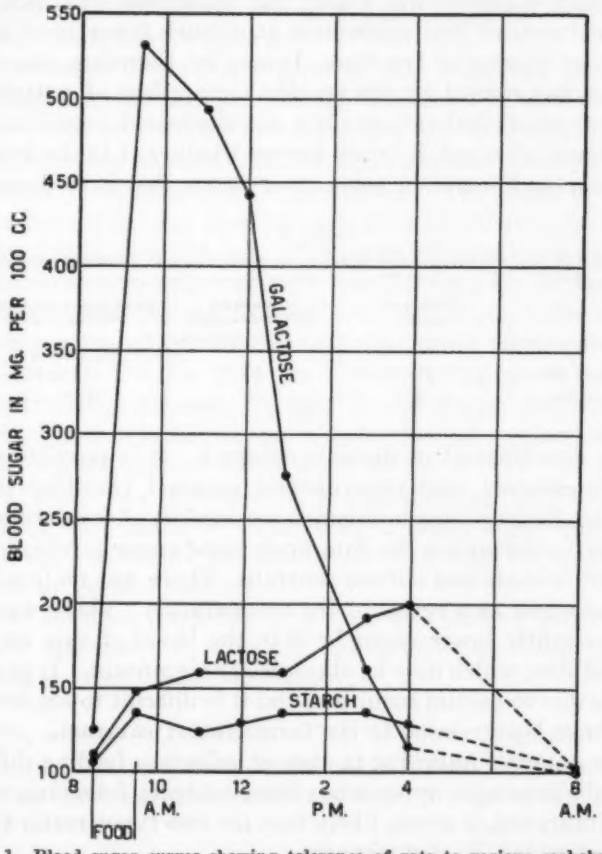

Fig. 1 Blood sugar curves showing tolerance of rats to various carbohydrates. After a preliminary 16-hour fast the animals were given diets containing the carbohydrates indicated on the curves. The animals were given access to a weighed quantity of food for 1 hour, after which it was withdrawn.

caused cataract in the shorter period (galactose) is also the carbohydrate that caused the higher levels of blood sugar. However, even if we were certain that the blood sugar was responsible for the production of cataract, it is of course impossible to state whether it was the level of blood sugar or the specific nature of the sugar that caused the cataract. Kirby, Estey and Wiener ('32) found that galactose was toxic to tissue cultures of lens epithelium at a much lower level than was either glucose or fructose. It may be, therefore, that the cataract was caused by the specific toxic effect of galactose. It is recognized that cataract is a not uncommon complication of diabetes, although it is not known whether it is the hyperglycemia, the lipemia, or some other factor that is responsible

TABLE 3

Blood sugar in rats receiving a diet deficient in vitamin G and a normal control diet

	NUMBER OF RATS	NUMBER OF DETERMINATIONS	MEAN BLOOD SUGAR WITH PROBABLE ERROR
			mg. per 100 cc.
Deficient diet	21	40	107 ± 1.2
Normal diet	9	38	118 ± 1.4

for the development of diabetic cataract. It is possible that diabetic cataract, and experimental cataract resulting from galactose feeding, have a common mechanism of formation.

Table 3 summarizes the data upon blood sugar in vitamin G deficient animals and normal controls. There was no increase in blood sugar as a result of the avitaminosis. There was, in fact, a slightly lower sugar level in the blood of rats on the deficient diet, which may be statistically significant. It probably was due to partial inanition, and it is difficult to see how it could have been related to the formation of cataract.

Since cataract following lactose or galactose feeding differs in ophthalmoscopic appearance from cataract following vitamin withdrawal, it seems likely that the two types result from different metabolic disturbances.

SUMMARY

Young growing rats were given adequate diets containing various carbohydrates as 60% of the diet. Blood sugar was determined by the Folin micro method. Seventeen out of eighteen rats receiving lactose developed cataract at an average time of 44 days. The three rats receiving galactose exhibited cataract on the eleventh day. Glucose, sucrose and starch did not cause cataract.

The mean blood sugar level in rats receiving glucose ad libitum was 121 mg. per 100 cc. The level in rats receiving starch was essentially the same, but was slightly higher in rats receiving sucrose. Lactose and galactose caused high and extremely variable blood sugar levels. The mean of eighty-nine determinations upon lactose rats was 160 mg.; the highest value found was 234 mg. The mean of twenty-seven determinations upon galactose rats was 372 mg.; the highest level recorded was 556 mg. Considered statistically, the chances are more than 1,000,000,000 to 1 that these mean values are significantly greater than the corresponding mean value for rats receiving glucose. It may be significant that the sugar that caused cataract in the shorter period (galactose) also produced the highest blood sugar levels.

Cataract was produced in young growing rats by a diet deficient in vitamin G. The mean of blood sugar determinations upon such rats was somewhat lower than the mean blood sugar of normal controls.

The cataract produced by lactose or galactose feeding was easily distinguished with the ophthalmoscope from cataract resulting from vitamin deficiency.

The author is indebted to Mr. William J. Darby for assistance in care of the animals and to Dr. K. W. Cosgrove for making slit-lamp examinations of eyes of some of the animals.

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COMPARISON OF THE BIOLOGICAL AND CHEMICAL METHODS FOR THE DETERMINATION OF VITAMIN C IN CANNED STRAINED VEGETABLES AND A STUDY OF ITS VARIATION FROM YEAR TO YEAR [1]

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For 5 years, we have studied the vitamin C content of canned strained vegetables of one brand: tomatoes, spinach, peas and green beans. For the first 2 years, the biological test on guinea pigs was used; then, for 2 years, the biological test was compared on the same foods with the chemical method of titrating with sodium 2:6 dichlorobenzenone indophenol; and, in 1935, the chemical method alone was used. This series of tests then gives data upon which to compare the two methods of determining vitamin C in these products and to note the variation in vitamin C potency from year to year.

I. COMPARISON OF METHODS

A chemical method, such as titrating the reducing value of an extract of the vegetable with sodium 2:6 dichlorobenzenone indophenol, is more sensitive and much less expensive and time consuming than the biological test. However, the titration method is not specific for vitamin C and hence must be carefully controlled and checked for its reliability. We used this method, at first, as a guide in choosing the quantities to be fed the guinea pigs, since the biological tests on such products in 1930 and 1932 showed great variation.

[1] Financed by a grant from the Gerber Products Co.

The volumetric method of Bessey and King ('33) was used with two minor changes. Instead of three grindings of the food with sand and 10% acetic acid, only one grinding was necessary for the strained foods, but four additional washes were made. Since many of the extracts were colored and the red color of the dye difficult to see, a distinct end point was made possible by choosing a pink color stable for 10 seconds as the end point and by comparing it with two blanks: one of the extract as titrated, and another to which 1 or 2 drops of methyl red had been added to give a stable color similar to the end point.

To determine whether this method was a reliable one for canned foods, we studied its accuracy and possible errors and compared the chemical and biological methods on eleven samples of canned strained vegetables. Each sample was analyzed in triplicate or quadruplicate. In a series of samples, a solution of pure vitamin C was added at the beginning of the analysis to one of the aliquots. From the titration values of the aliquots and of the solution of vitamin C, the per cent of recovery was calculated. The results averaged 95.75%, ranging from 91.2 to 97.2%. It was thought possible that iron and tin salts dissolved from the can would interfere with a true test. However, the addition of the maximal amounts of tin and iron salts found in canned foods to some of the aliquots being titrated did not change the values. Concentrations of oxalic acid as found in spinach, likewise, did not affect the results. Aliquots from the same sample ordinarily checked within 3 to 5% error, even when small amounts of vitamin C were present. The method, thus, could be considered of greater sensitivity and accuracy than a method based on an animal response, but whether vitamin C was truly measured needed to be checked by comparison with the biological method.

The biological method of Sherman, LaMer and Campbell ('22) was used with an addition of 2% dried baker's yeast and 2% cod liver oil to the basal diet. The data of all animals which consumed the supplements are included in the averages. Some of the tests were exploratory in nature and only a few

animals used. The control animals, those fed cabbage or lemon juice or the basal ration only, were quite uniform from year to year, as shown in table 1. As the International Standard, 1 cc. lemon juice contains 10 units and gives an average scurvy score of 1 to 2. The vegetable supplement giving scurvy scores approximately the same would contain 10 units, or a score of 0, as from 1.5 cc. of lemon juice, indicates 15 International units and may be used for comparisons also.

To compare the values obtained by the chemical and biological methods, the analytic data from the titration values

TABLE 1

Record of control animals in the biological vitamin C determinations

SUPPLEMENT	YEAR	AMOUNT FED	NUMBER OF ANIMALS	AVERAGE INITIAL WEIGHT	AVERAGE GAIN IN WEIGHT	AVERAGE SURVIVAL TIME[1]	AVERAGE SCURVY SCORE	RANGE OF SCORE
		cc.		*gm.*	*gm.*	*days*		
None	1930		6	316.2	— 99.0	30	15.0	9–17
None	1932		5	299.8	— 97.6	29	13.4	7–21
None	1933		5	329.0	— 86.0	27	13.6	10–17
None	1934		6	321.0	— 109.8	25	15.8	10–21
Raw cabbage	1930	Ad lib.	4	301.5	+ 312.0	90	0	0
Lemon juice	1932	1.5	3	282.3	+ 255.0	90	0	0
Lemon juice	1932	1.0	7	329.0	+ 172.6	90	1.2	0–4
Lemon juice	1933	1.0	9	310.3	+ 153.8	56	1.8	0–3
Lemon juice	1934	1.0	9	289.0	+ 154.8	56	1.4	0–4

[1] The experimental test period of 1933 and 1934 was limited to 56 days, but for 1930 and 1932 was 90 days.

of the vegetables of the years 1933 and 1934 were used in calculating the amounts to be fed the guinea pigs. A series of lemons were tested and found to contain on the average 0.565 mg. vitamin C per cubic centimeter, equivalent to 10 International units. The guinea pigs were fed sufficient of each vegetable to give 0.565 mg. vitamin C, based on its titration value. The data are presented in table 2. It will be noted that of the eleven samples from the 2 years the average scurvy scores for the vegetable supplements ranged from 0.7 to 2.4 while those of 1 cc. lemon juice in table 1 were 1.2 to 1.8.

Considering deviations found in the chemical method, the usual biological variations of the animals and the more or less qualitative scoring of the degree of scurvy, the data indicate that the two methods give reasonably concordant results. Since the chemical method gave the same result as the biological, the vegetables canned in 1935 were tested chemically only.

TABLE 2

Record of animals fed vegetable supplements containing equal quantities of vitamin C[1]

SUPPLEMENT	YEAR	AMOUNT FED	NUMBER OF ANIMALS	AVERAGE INITIAL WEIGHT	AVERAGE GAIN IN WEIGHT	AVERAGE SCURVY SCORE	RANGE OF SCORE
		gm.		*gm.*	*gm.*		
Tomatoes	1933	4.1	7	340.4	+ 96.6	0.7	0–2
Tomatoes A		4.1	7	336.7	+ 123.7	1.0	0–4
Tomatoes B		4.3	8	290.9	+ 180.9	1.1	0–3
Tomatoes C		9.0	8	294.3	+ 137.0	2.3	0–6
Spring spinach[2]	1933	4.3	7	301.7	+ 131.6	1.6	1–2
Spring spinach	1934	5.7	7	308.4	+ 112.7	2.4	1–4
Fall spinach	1934	3.4	9	301.0	+ 154.9	2.3	1–5
Peas	1933	6.3	8	317.3	+ 239.8	1.4	0–3
Peas	1934	8.5	8	308.4	+ 181.0	1.9	0–4
Green beans[2]	1933	12.5	5	300.2	+ 147.9	2.2	2–3
Green beans	1934	17.8	8	317.8	+ 195.4	2.0	1–3

[1] These vegetables were first tested chemically and the quantities fed calculated from that data to contain 0.565 mg. vitamin C.

[2] The animals receiving spinach and green beans had to be coaxed and sometimes fed by hand but the supplements were completely consumed.

II. VARIATION IN VITAMIN C CONTENT FROM YEAR TO YEAR

The samples used for all these tests were representative of the entire season's pack except for tomatoes A, B and C which were special samples and for the 1930 vegetables which were chosen from the warehouse. These foods were canned by the Gerber Products Co. who dated and set aside cans from each day's pack. An equal number of cans of each date were taken to form the twenty-four to sixty cans required for a representative sample. The error in sampling was, thus, practically eliminated.

The results of determinations, both chemical and biological, are presented in table 3 and calculated in terms of International units per ounce. The unit value is determined in the

TABLE 3

The variation in vitamin C content of canned strained vegetables from year to year

SUPPLEMENT	YEAR	TOTAL SOLIDS[1]	NUMBER OF ANIMALS	AMOUNT FED	AVERAGE SCURVY SCORE	NUMBER OF SAMPLES FOR CHEMICAL DETERMINATION	TITRATION VALUE	VITAMIN C
		%		gm.			*mg. vitamin C. per gram*	*International units per ounce*[2]
Tomatoes	1930	12.30	5	3.0	0.0	0		142
Tomatoes	1932	14.80	4	2.0	13.8	0		†[3]
Tomatoes	1933	13.96	7	4.1	0.7	4	0.137	70
Tomatoes	1935	12.27	0			4	0.129	64
Spinach	1930	5.60	2	12.0	1.5	0		24
Fall spinach	1931	6.93	6	16.0	0.0	0		27
Spring spinach	1932	6.59	6	16.0	0.8	0		18
Spring spinach	1933	7.89	7	4.3	1.6	5	0.132	66
Spring spinach	1934	7.27	7	5.7	2.4	4	0.099	50
Fall spinach	1934	7.47	9	3.4	2.3	4	0.167	85
Spring spinach	1935	6.72	0			2	0.104	53
Peas	1930	12.10	4	8.0	2.4	0		35
Peas	1932	12.71	4	8.0	0.0	0		53
Peas	1933	14.47	8	6.3	1.4	4	0.090	45
Peas	1934	13.44	8	8.5	1.9	4	0.066	33
Peas	1935	13.38	0			4	0.063	32
Green beans	1930	6.08	2	16.0	4.0	0		< 17
Green beans	1932	6.81	6	16.0	0.7	0		17
Green beans	1933	7.45	5	12.5	2.2	3	0.045	23
Green beans	1934	6.56	8	17.8	2.0	4	0.032	15
Green beans	1935	6.33	0			4	0.018	9

[1] Determined in a vacuum oven at 70°C. except for fall spinach of 1931 and 1934 which were air dried in an electric oven at 100°C. to constant weight. Attention is called to the fact that while the total solids content of the canned strained spinach was approximately the same as for raw spinach, the tomatoes contained twice and both peas and green beans about one-half the average total solids content of raw vegetables.

[2] These values are based on either biological test when no titration value was determined or upon the chemical method when the titration value is given.

[3] This value was too low to be calculated since none of the control animals receiving lemon juice had scurvy scores approximating this. The animals did live through the experimental period while the negative controls with similar scores did not.

biological test by comparison with the scurvy scores of guinea pigs fed lemon juice or, in the chemical method, by the amount which from the titration data contains 0.0565 mg. of vitamin C.

The potency of these products varies markedly from year to year as noted in the last column of table 3. Tomatoes were high in vitamin C in 1930 but very low in 1932, for which we have no explanation except that of seasonal variation in the raw product. None were canned in 1934 since the raw product was inferior. The canning method was greatly changed in 1933 by using a new type of cooker for evaporation, and in 1935 a marked reduction in the time of heating. The tomatoes contained 70 International units vitamin C per ounce in 1933 and 64 in 1935. Thus, there was no increase in vitamin C content due to the change in method which the seasonal variation in raw tomatoes did not mask.

Spinach has been especially rich in vitamin C for the last 3 years, particularly in the fall of 1934, and shows a marked improvement over that of the 3 earlier years. A steam blanch has been used since 1933 and the evaporation time reduced. The 'shaker-cooker' process was introduced in 1934. Spinach was found to vary from 18 to 27 International units vitamin C per ounce from 1930 to 1932 but 50 to 85 units from 1933 to 1935.

Peas showed a variation in vitamin C content from 32 to 53 International units per ounce. The total solids content has been increased since 1933 by greater evaporation and the 'shaker-cooker' used for the final heating since 1934. It is interesting to note that peas are more uniform in vitamin C from year to year than the other vegetables.

Green beans are a fair source of vitamin C, varying from 9 to 23 International units per ounce. The 'shaker-cooker' process was introduced in 1934. The method was not changed from 1934 to 1935 yet the last year's pack was low in vitamin C.

A study of the variation in vitamin C content in these four vegetables shows that it is difficult to indicate which factors had the greatest effect on their potencies. Since all of these vegetables during the last 4 years were carefully chosen as

representative samples, the error of sampling is practically nil. There were some differences in total solids content from year to year but they are not significant in explaining the vitamin variation. The soil conditions were fairly uniform and there were very few changes in varieties of plants. Although the raw foods were carefully graded as to appearance and degree of maturity, there seems to be an important seasonal variation.

Another factor which may influence the final antiscorbutic potency is that of factory procedure. The method used depends on exclusion of air by a blanket of steam or closed container and has been modified, as previously indicated, in several ways to reduce heating time. The factory method has been gradually improved from year to year so that we should expect any losses of vitamin C to be steadily reduced. Although the data do not clearly distinguish the effect of the two factors, the original variation in raw foods due to weather conditions and the losses in the factory process, yet it seems to us that, in this study, the seasonal variation is the more important.

It is evident that predictions of potency from 1 year's test may be far different from that actually found in another year, and that cans chosen from the grocer's shelf may vary widely in vitamin C potency. The producer of canned strained vegetables must be diligent in considering all the factors affecting their vitamin C potency and carefully controlling those within his power in order that a product as rich as possible in vitamin C be placed upon the market. The standardization of such potency, however, from the standpoint of the manufacturer, presents a difficult problem.

SUMMARY

The determination of vitamin C in canned strained vegetables by titration with sodium 2:6 dichlorobenzenone indophenol has been found accurate and reasonably concordant with biological tests on the same samples.

Marked variations in vitamin C content have been found in tomatoes, spinach, peas and green beans from year to year.

A discussion of the factors affecting the quality of the vegetables leads to the conclusion that the standardization of the vitamin C potency even under carefully controlled conditions is a difficult problem.

The author wishes to thank Phillip Baker, Jr., Charles Noble, Lorraine Rauls and John Vucich for assistance in the vitamin C determinations and Ray B. Wakefield for the total solids determinations.

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STORAGE OF VITAMIN C BY NORMAL ADULTS FOLLOWING A PERIOD OF LOW INTAKE [1]

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ONE FIGURE

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It is recognized that the human body has some ability to store vitamin C (Yavorsky, Almaden and King, '34; Johnson and Zilva, '34), therefore the apparent requirement at any given time will depend in part on the store of vitamin C in the tissues. It is also recognized that the urinary excretion of ascorbic acid is dependent upon at least two factors: 1) the dietary intake and 2) the reserves of vitamin C in the tissues (Johnson and Zilva, '34; Harris and Ray, '35). With this in mind it seemed desirable to investigate the urinary output of persons in a known state of saturation with respect to vitamin C.

Since it has been reported (Göthlin, '31; Dalldorf and Russell, '35) that capillary resistance varies with the vitamin C intake, it was hoped that change in capillary resistance would serve as an indication of vitamin C subnutrition in this study.

EXPERIMENTAL

Urinary excretion. This experiment was planned to determine the vitamin C intake necessary to reestablish saturation after prolonged administration of a low C diet.

[1] The manuscript from which these data were taken was submitted by Patricia H. O'Hara in partial fulfillment of the requirements for the degree of master of science, 1936. The authors wish to express their appreciation to Dr. Helen Bull, who made the medical examinations, and to the subjects without whose cooperation the study would have been impossible.

The vitamin C content of foods and urine specimens was determined by indophenol titration essentially according to the method of Bessey and King ('33). Ten cubic centimeters of urine were diluted to 50 cc. with 8% acetic acid, in a volumetric flask, and 10 cc. of this solution were titrated rapidly against a standard solution of 2-6 dichlorophenolindophenol from a microburette. Preliminary trials indicated that a more satisfactory end point was obtained, especially in dilute solution, by this method than when the reverse titration was employed as suggested by Birch, Harris and Ray ('33).

During the day time urine specimens were titrated immediately after voiding. The evening and first morning specimens were preserved by acidifying with 10% glacial acetic acid as suggested by Harris and Ray ('35) and titrated in the morning. Titrations made on the same specimen when freshly voided and after preserving in this way from 2 to 12 hours, confirm the observation of Harris and Ray that little loss occurs. In addition, since most of the vitamin C ingested in this experiment was ingested with the morning meal, and since the peak of excretion was reached in 4 to 6 hours after ingestion of a large dose of vitamin C, the error introduced by preserving the evening and first morning specimens was certainly small and does not invalidate the results of the study.

In a preliminary experiment the two subjects were saturated as a result of the inclusion of liberal amounts of orange juice in the diet. Weekly determinations on their usual diets indicated an average excretion of about 70 and 77 mg. of ascorbic acid, respectively.

On the basal diet which provided approximately 11 mg. of ascorbic acid per day, the urinary excretion fell to an average of 11 and 17 mg. during the second week. When orange juice was again included in the dietary approximately 700 and 900 mg. of ascorbic acid were ingested, over a period of 8 to 9 days, before any rise in urinary excretion occurred. This experiment was discontinued before the subjects reached saturation.

In the present experiment, four normal adult women were used as subjects. Titrations made previous to the experiment while the subjects were on their usual diets, indicated that their tissue reserves of vitamin C were satisfactory, since the average urinary excretion of ascorbic acid ranged from 65 to 77 mg. per day.

The basal diet was planned to be adequate with respect to all other nutritive factors, but contained only 5 mg. of vitamin C. The following foods were used ad libitum: a whole wheat cereal with added wheat germ, butter, brown sugar, Ry Krisp, whole wheat and white flour, nuts, cooking fat, eggs, rice, coffee and postum. The following were included in definite amounts each day, either because they contained traces of vitamin C, or because a certain intake was considered advisable to insure the adequacy of the diet: ¼ cup evaporated milk, 2 ounces American cheese, 100 gm. canned carrots, 50 gm. cooked dried prunes, 100 gm. cooked frosted squash, 100 gm. canned pears, 100 gm. hamburger steak. The approximate average composition of the basal diet, as estimated from tables of food composition was as follows: protein, 83 gm.; calcium, 1.04 gm.; phosphorus, 1.50 gm.; iron, 12 mg.; calories 2240. The orange juice additions were titrated daily, immediately before the dose was given.

The experiment was divided into three periods as follows:

I. A preliminary period (4 to 5 days) to be sure that all subjects were saturated. In addition to the basal diet orange juice of known potency was ingested as indicated in figure 1.

II. Basal diet only (29 to 30 days).

III. Basal diet plus orange juice with an ascorbic acid content of 200 mg., taken at breakfast. This period was continued until the subjects were saturated, as evidenced by failure to show further increases in urinary output of vitamin C on a constant high intake (15 to 17 days).

Capillary resistance studies. Capillary resistance was determined weekly, alternately on the right and left arms, by Göthlin's method ('31) and daily by means of the resistometer devised by Dalldorf (Dalldorf and Russell, '35).

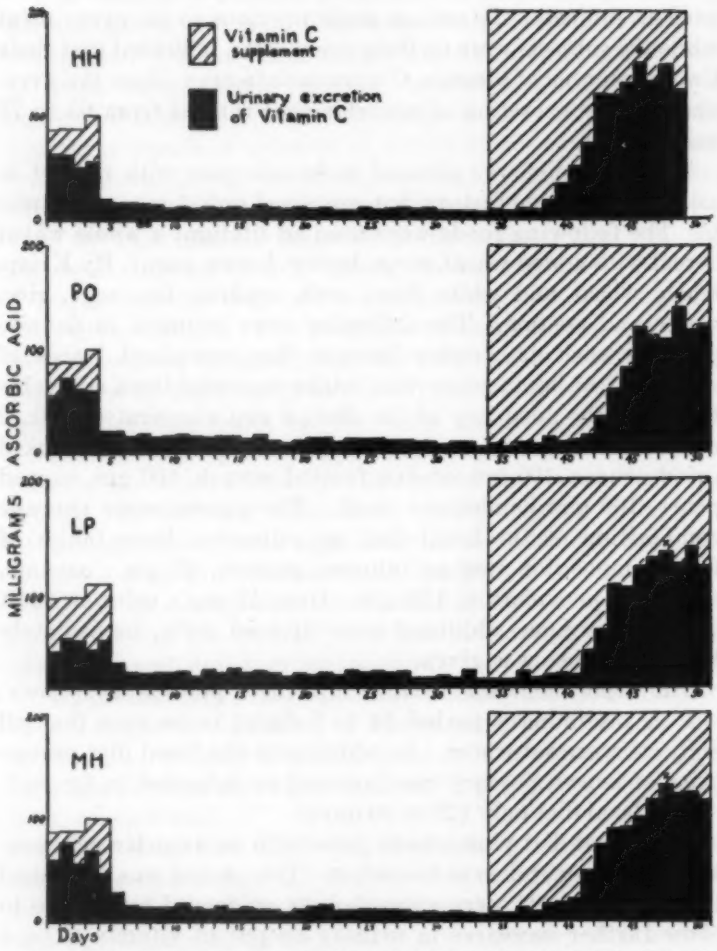

Fig. 1 This figure shows the relation of daily intake of vitamin C to the 24-hour excretion of ascorbic acid. A basal diet providing 5 mg. of vitamin C was used throughout. X indicates the day on which the subject was considered to be saturated when a vitamin C supplement was resumed after a period of deprivation.

In consideration of the factors which Göthlin ('33) has found to influence capillary resistance, all tests were carried out at comfortable room temperature, after the subjects had been indoors for an hour or more. Subjects H.H. and P.O. took a warm shower each morning, while the other subjects took tub baths in the evening. All tests were carried out between 9 A.M. and 2 P.M.

Göthlin's standard ('31) for judging the response was used; the resistance was considered normal when less than five petechiae occurred at a pressure of 50 mm. mercury, and subnormal when more than eight occurred at this pressure, or when two or more petechiae appeared at 35 mm. pressure.

In determining capillary resistance with the Dalldorf resistometer, tests were made in an area 3½ inches square, on the right outer arm, midway between shoulder and elbow. Twenty-five tests could be made in this area, so that a given space would not be retested for 5 or 6 days.

In reading the test, the surrounding tissue was blanched by pressing with a plane glass, thus bringing petechiae into prominence. All readings were made by a daylight lamp.

On days when the Göthlin test was made on the right arm, the determination was made first by the method of negative pressure to avoid possible influence of the venous stasis produced during the Göthlin test.

In the preliminary experiment, readings made with Dalldorf's resistometer were considered positive if a single hemorrhage was observed. The readings so obtained were consistently lower than those reported by Dalldorf and Russell ('35) for well-nourished persons. Later,[2] it was decided to adopt as the threshold reading the lowest negative pressure which, on casual inspection, gave an immediate impression that bleeding had occurred.

In addition to the impression as to whether a reading was 'positive' or 'negative,' the space tested, the number and size of petechiae, and the appearance of the area on the following day were recorded. The readings which were considered positive were characterized by the appearance of several petechiae

[2] On the basis of a conference with Dr. Gilbert Dalldorf.

(about 80% included seven to twenty-five petechiae) distributed through the test space (not confined to the border), producing a discolored area which was apparent the following day. As a rule, in readings which were considered negative, there were from none to ten petechiae.

RESULTS AND DISCUSSION

Urinary excretion studies. Figure 1 shows the dietary intake and urinary excretion of ascorbic acid by the four subjects.

It has been shown (Abassy, Harris, Ray and Marrack, '35) that the non-specific reduction titre, due to substances other than vitamin C, does not normally rise above an equivalent of 3 to 6 mg. per day. In the accompanying tables, the values are uncorrected.

It will be noted that the subjects were 'saturated' at the beginning of the experiment, i.e., on an intake considerably higher than the estimated requirement, the subjects maintained a relatively constant output. If unsaturated, the subject requires several days to reach a relatively constant output (Johnson and Zilva, '34). During the period on the basal diet only, the urinary output of ascorbic acid fell sharply, reaching a low level of 15 mg. in 1 to 2 days.

It is of interest that this fall in urinary excretion of vitamin C occurred less rapidly and was less marked in the preliminary experiment, in which the basal diet provided approximately 11 mg. of ascorbic acid per day, than in this experiment when the basal diet contained approximately 5 mg. of ascorbic acid. It is possible that the continued excretion of the usual amounts of ascorbic acid which has been observed by some workers (Harris, Ray and Ward, '33; Hawley, Stephens and Anderson, '36) may be due to the use of a basal diet more liberal in vitamin C. Hawley, Stephens and Anderson estimated the ascorbic acid content of their low vitamin C diet as between 10 and 20 mg. per day.

Table 1 summarizes the ascorbic acid excretion during the preliminary saturation period and the period of vitamin C

deprivation. All subjects showed a general tendency to decreasing excretion of ascorbic acid as the period on the basal diet only progressed. Indeed this tendency continued for a few days after the orange juice supplement was resumed. At the end of a month on the basal diet all subjects continued to excrete indophenol reducing substance over and above the 3 to 6 mg. estimated by Abassy, Harris, Ray and Marrack ('35) as representing the non-specific titration. It was not, of course,

TABLE 1

Summary of ascorbic acid excretion on a diet low in vitamin C, following saturation

PERIOD	DAYS OF PERIOD	ASCORBIC ACID IN URINE							
		H.H.		P.O.		L.P.		M.H.	
		Range	Average	Range	Average	Range	Average	Range	Average
I		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Ascorbic acid supplement 88–100 mg.	1–4 or 5	50–63	56	56–72	62	34–48[1]	42	46–77[1]	61
II	1		19		21		24		28
Basal diet	2– 8	11–15	13	14–21	16	11–17	15	12–16	14
	9–15	9–13	11	10–16	14	11–18	14	10–14	12
	16–22	8–11	9	11–14	13	12–15	13	11–15	12
	23–29 Or 30	7–11	9[2]	6–12	10[2]	9–14	11	7–11	9

[1] Five-day period.
[2] Thirty-day period.

intended that the experiment should continue until clinical signs of scurvy should appear.

That the bodily stores of vitamin C were nearing depletion is suggested by the fact that in one subject (H.H.) the gums on the left side of the mouth, on both upper and lower jaws from the first premolars through the second molars were swollen, edematous and purple. Since this occurred during the fourth week on the low C diet, and disappeared quite promptly after orange juice was administered, it may probably be interpreted as a symptom of vitamin C subnutrition.

All subjects were examined by a physician at the end of the second period. Particular attention was paid to examination for symptoms of latent scurvy: tenderness over the long bones, edema and hemorrhage of the gums and roof of mouth, abnormal heart sounds, hemorrhages in skin and conjunctiva, and red blood cells in the urine. With the exception of the gingival bleeding observed in the subject H.H., no clinical symptoms suggestive of vitamin C subnutrition were found. It is of interest that in this subject, the urinary excretion of vitamin C fell more rapidly on the basal diet than in the other subjects (fig. 1) reaching a relatively constant low level of excretion in the third week. Furthermore this subject showed a more rapid rise in excretion when vitamin C was again included in the diet, and reached saturation earlier than the other subjects. Whether this difference was due to the smaller body size of the subject H.H. or to an individual difference in capacity to store the vitamin, or to some other factor, it is impossible to conclude from these experiments.

During saturation on the 200 mg. supplement, the average variation in urinary excretion from that which occurred on the day saturation was reached was only 11 mg., or less than 6%. The maximum variation during saturation ranged from 7.5 to 13% for the four subjects.

There was a general tendency for the difference between intake and urinary excretion to be greater when equilibrium was established on an intake of 200 mg. than on the lower intake of the first period. At present all of the factors which cause this difference between intake and urinary excretion are not known. In addition to storage, it may represent failure of absorption, excretion by paths other than the kidney, destruction in the bladder, chemical changes during metabolism and other factors.

However, unless some ability to synthesize vitamin C is assumed, one may say that the amount of vitamin C stored on a given day cannot be more than the difference between intake and urinary output. Therefore a study of these differences, following vitamin C deprivation, up to the time saturation is reached, should give some indication of possible storage.

Table 2 summarizes the ascorbic acid excretion when orange juice was given, following a period of vitamin C deprivation. From 1000 to 1600 mg. were ingested before a noticeable increase in excretion occurred. From 2200 to 2800 mg. had been taken before saturation was reached. Of this, from 1710 to 2181 mg. was not accounted for in the urine. It seems probable that a large part of this difference was stored. It has been shown that the store of vitamin C in the guinea pig liver is reduced to about one-third when the guinea pig begins to lose weight on a scorbutic diet, before manifest symptoms of scurvy

TABLE 2

Summary of urinary excretion of ascorbic acid, in relation to intake, following Vitamin C deprivation

SUBJECT	H.H.	P.O.	L.P.	M.H.
From beginning of period to first distinct increase in ascorbic acid excretion				
Number of days	5	8	8	6
Total amount supplement, mg.	1000	1600	1600	1200
Total ascorbic acid output, mg.	57	95	120	75
Difference between intake and output, mg.	943	1505	1480	1125
From beginning of period to saturation				
Number of days	11	14	13	13
Total amount supplement, mg.	2200	2800	2600	2600
Total ascorbic acid output, mg.	490	619	617	620
Difference between intake and output, mg.	1710	2181	1983	1980

appear (Yavorsky, Almaden and King, '34). If we assume a similar store remaining in the tissues of these subjects after a month on a low C diet, the resulting estimation of the storage of these subjects at saturation would be in the neighborhood of 2500 to 3000 mg.

It is unfortunate that of the considerable number of studies on urinary excretion of ascorbic acid in the literature most yield no information on the amount of ascorbic acid which might represent storage at saturation, either because nothing was known of the state of vitamin C reserves at the outset, or the experiments were not carried on to saturation, or the doses were varied so that the probable date of saturation could not

be estimated, or because the data on urinary excretion were incomplete.

Johnson and Zilva ('34) found that a man who had been on a winter diet (England) and whose excretion of ascorbic acid was so low as to be almost negligible, reached saturation when 1485 mg. of ascorbic acid had been ingested. Of this amount 1141 mg. did not appear in the urine. Although the variation in daily dose makes it difficult to know just when their subject A.E.K. became saturated, these authors found that saturation occurred more quickly on a high intake—apparently after 3649 mg. had been ingested during a 4-day period. Of this 2363 mg. did not appear in the urine. Harris and Ray ('35) had one subject with a daily excretion of about 20 mg. of vitamin C on his usual diet, whose ascorbic acid excretion was followed after 8 days on a vitamin C free diet until he became saturated. In the course of a 25-day period (including 7 days on a low C diet) this subject ingested 3500 mg. of ascorbic acid, of which 1060 appeared in the urine, leaving a balance of 2440 mg. unaccounted for.

The excretion during a period on a diet low in vitamin C is not an indication of the extent to which body stores are being depleted, since in the experiments here reported, the difference between vitamin C intake and excretion up to the time saturation was reached, was more than five times the ascorbic acid excretion during the low C period which preceded.

In addition to the studies on living subjects, the study of Yavorsky, Almaden and King ('34) on tissues obtained at autopsy give a clue to the storage of vitamin C in the body. On the basis of their figures for the average vitamin C content of human tissues (adrenal, brain, heart, liver, pancreas, kidney, lungs, spleen), the average store of vitamin C in the adult would be in the neighborhood of 850 to 950 mg. However, these authors state that the maximum concentration found was approximately three times the average, which would lead one to expect a maximum storage of approximately 2500 to 2850 mg. These figures agree closely with the estimated storage on the basis of the present study.

It is of interest to see how the data obtained in this experiment fit the several methods which have been suggested for estimating the state of the vitamin C reserves in the human body. Archer and Graham ('36) believe that special significance attaches to the amount of ascorbic acid which must be taken before the output rises above 75%. In the case of the four subjects here reported, the average percentage output ranged between 53 and 72 during saturation at different levels of intake. On only 1 day, did the excretion for any subject rise to 75% during saturation on the 200 mg. level; the average excretion for the four subjects on this level varied from 62 to 70% of the intake. During the preliminary saturation period two other subjects reached an excretion of over 75% on 1 day each.

Harris and Ray ('35) state that with the usual volume of urine, an adult excreting 0.02 to 0.03 mg. per cubic centimeter per day may be safely assumed to have adequate vitamin C reserves, although these authors consider the response to a large test dose to be a better criterion.

Ippen ('35) suggests that a rise in urinary excretion to more than 0.04 mg. per cubic centimeter after a large test dose may be used as an indication of satisfactory reserves of vitamin C.

In the present studies the ascorbic acid excretion fell below 0.02 mg. per cubic centimeter per 24 hours within a day or 2 after the low vitamin C period was begun and did not return to this level until 5 to 9 days after the 200 mg. supplement had been added. At this time the total daily urinary excretion of vitamin C ranged from 18 to 36 mg. for the four subjects. The concentration of vitamin C in the urine following the ingestion of a 200 mg. dose rose to 0.04 mg. per cubic centimeter at the height of the excretion either coincidentally with or the day following that on which the total daily excretion reached 0.02 mg. per cubic centimeter. Thus it appears that these two criteria measure a similar state of vitamin C reserve, and that they may be met when the body falls considerably short of saturation.

Capillary resistance studies. The results of the capillary studies for a consecutive period of 14 to 17 weeks are summarized in table 3. It is apparent that there were marked variations in response to the capillary resistance tests, not only in different individuals, but in the same subjects from time to time. There was more variation between maximum and minimum readings obtained by the Dalldorf method during a given period, than between the average readings for the several periods.

Frequently both negative and positive readings were obtained at the same pressure in successive tests on the same day. Of ninety-four such tests on the four subjects, seventy-seven or 82% of the negative readings occurred in the two-fifths of the area nearest the elbow. In general, it appeared that higher pressures were required to produce hemorrhage in the spaces toward the elbow, than those toward the shoulder.

With Göthlin's test, some borderline and subnormal readings were obtained in all subjects on their usual diets. These were particularly frequent in subject H.H., who, judging from the urinary excretion of ascorbic acid on her usual diet, must have had satisfactory tissue reserves. Of eighteen subnormal tests obtained in fifty-eight determinations by Göthlin's method on the four subjects, eight occurred during the period of low vitamin C intake, five during the recovery period (three when the subjects were saturated or nearly so) and five on the subject's usual diets. Of a total of sixteen tests made during the period on the basal diet, nine were normal, eight subnormal and three borderline according to Göthlin's standard.

Of twelve tests made during menstrual periods, six gave subnormal values. One of these occurred during the period of vitamin C deprivation.

No correlation was found between the results obtained by the two methods. Subjects L.P. and M.H., whose capillary resistance as judged by the Dalldorf test tended to be low, had fewer subnormal or borderline tests as judged by Göthlin's technic than the other subjects. Many examples of lack of correspondence between the results of the two tests were

TABLE 3

Relation of capillary resistance to vitamin C intake and excretion

DIET AND PERIOD	H.H.					P.O.					L.P.					M.H.				
	Capillary resistance				Ascorbic acid in urine, mg.	Capillary resistance				Ascorbic acid in urine, mg.	Capillary resistance				Ascorbic acid in urine, mg.	Capillary resistance				Ascorbic acid in urine, mg.
	Göthlin's method		Dalldorf's method			Göthlin's method		Dalldorf's method			Göthlin's method		Dalldorf's method			Göthlin's method		Dalldorf's method		
	35 mm.	50 mm.	cm.	Range for week, cm.		35 mm.	50 mm.	cm.	Range for week, cm.		35 mm.	50 mm.	cm.	Range for week, cm.		35 mm.	50 mm.	cm.	Range for week, cm.	
Usual diet	0	1	30	20–30	99	0	3	40		63										
Usual diet	0	5	30	25–30	66	0	4	35	25–35	37										
Usual diet	0	3	35	20–35	84	1	3	45	30–45	91										
Usual diet	0	2	54	20–54	65	*5*	8	40	30–45	89	0	1	25	....	65	0	2	..	....	65
Basal diet																				
2nd day	*2*	*10*	15	15–20	15	0	4	30	30–35	21	*2*	4	15	10–20	24	0	1	20	10–20	28
9th day	0	*10*	15	15–25	12	*2*	5	25	20–40	14	0	3	15	10–15	17	0	5	10	10–15	12
16th day	*2*	8	15	15–20	11	0	3	40	25–40	12	0	2	20	10–20	13	*2*	5	15	10–15	12
23rd day	0	8	15	15–20	12	0	7	25	25–35	10	0	1	10	10–15	13	0	4	15	10–20	12
30th day	1	4	20	15–20	7	0	3	30	25–40	6	0	*8*	15	10–20	9	0	*9*	10	10–20	7
Recovery period																				
7th day	0	*9*	20	10–25	52	*6*	*12*	25	25–40	13	1	1	15	10–15	22	0	3	15	15–20	13
14th day	*3*	5	20	10–25	149	*4*	8	30	25–40	110	*4*	6	15	10–20	132	0	6	15	10–15	132
Usual diet	1	1	20	20–30	70	*2*	*9*	30	30–35	21	1	1	15		20	0	4	15		20
Usual diet	*5*	5	15	....	139	0	1	25	25–35	20	*2*	3	15	10–15	18	0	5	15	15–20	16
Usual diet	1	4	20	....	105	1	3	40	25–40	14	1	3	20	....	16	1	4	10	10–15	13
Usual diet	1	7	25	....	135	1	3	30	30–35	15	1	2	15	....	19	0	0	20	15–20	35
Usual diet	0	4	20	15–25	98	0	5	25	25–35	15	0	0	15	....	19	1	6	20	15–20	18
Usual diet	*2*	8	20	....	159	1	8	30	....	24	1	1	15	....	37	0	3	20	....	32

During the period on the basal diet and the recovery period, the capillary resistance tests and determinations of urinary excretion of ascorbic acid were made on the same day; on the usual diet, determinations were sometimes 2 or 3 days apart. The readings which are in italics are subnormal according to Göthlin's standard.

noted. For instance, on the normal diet just before the experiment when P.O. showed five petechiae at 35 mm. and eight petechiae at 50 mm. pressure by Göthlin's method, the reading by Dalldorf's technic was 40 cm. On another occasion, when this subject had only 1 minute hemorrhage when tested by Göthlin's method at 50 mm. pressure, the reading by Dalldorf's technic was 25 cm.

It is evident that the data on capillary resistance do not show a correlation with urinary excretion of vitamin C.

In these experiments, it cannot be said that the determination of capillary resistance by either method gave an adequate indication of the state of nutrition in regard to vitamin C.

Variations in capillary resistance could not be correlated with the menstrual period.

SUMMARY

In four cases, the amount of vitamin C necessary to restore the tissues to saturation after a month on a diet very low in vitamin C, ranged from 2200 to 2800 mg., when administered in daily doses of 200 mg.

It is suggested that the difference between intake and excretion up to the point of saturation following prolonged deprivation of vitamin C may afford some indication of the maximum vitamin C reserve. On this basis tissue reserves at saturation appear to be of the order of 2500 to 3000 mg.

In these experiments, capillary resistance did not give an adequate indication of the state of nutrition with respect to vitamin C.

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